

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 December 2000 (14.12.2000)

PCT

(10) International Publication Number  
**WO 00/75319 A1**

(51) International Patent Classification<sup>7</sup>: C12N 15/12, 15/62, 5/10, C07K 14/71, A61K 38/17, A61P 43/00

(74) Agents: PALLADINO, Linda, O.; Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591 et al. (US).

(21) International Application Number: PCT/US00/14142

(22) International Filing Date: 23 May 2000 (23.05.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/138,133 8 June 1999 (08.06.1999) US

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707 (US).

**Published:**

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): PAPADOPOULOS, Nicholas, J. [US/US]; 2 Hillier Court, Butler, NJ 07405 (US). DAVIS, Samuel [US/US]; 332 W. 88th Street #B2, New York, NY 10024 (US). YANCOPOULOS, George, D. [US/US]; 1519 Baptist Church Road, Yorktown Heights, NY 10598 (US).



**WO 00/75319 A1**

(54) Title: MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES

(57) Abstract: Modified chimeric polypeptides with improved pharmacokinetics are disclosed. Specifically, modified chimeric Flt1 receptor polypeptides that have been modified in such a way as to improve their pharmacokinetic profile are disclosed. Also disclosed are methods of making and using the modified polypeptides including but not limited to using the modified polypeptides to decrease or inhibit plasma leakage and/or vascular permeability in a mammal.

MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED  
PHARMACOKINETIC PROPERTIES

5 The application claims priority of U.S. Provisional Application No. 60/138,133, filed on June 8, 1999. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

10

**INTRODUCTION**

The field of this invention is modified polypeptides with improved pharmacokinetics. Specifically, the field of this invention relates to  
15 Flt1 receptor polypeptides that have been modified in such a way as to improve their pharmacokinetic profile. The field of this invention also relates to methods of making and using the modified polypeptides including but not limited to using the modified polypeptides to decrease or inhibit plasma leakage and/or vascular permeability in a mammal.

20

**BACKGROUND**

The ability of polypeptide ligands to bind to cells and thereby elicit a phenotypic response such as cell growth, survival, cell product  
25 secretion, or differentiation is often mediated through transmembrane receptors on the cells. The extracellular domain of such receptors (i.e. that portion of the receptor that is displayed on the surface of the cell) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand binding characteristic. Binding of a ligand

to the extracellular domain generally results in signal transduction which transmits a biological signal to intracellular targets. Often, this signal transduction acts via a catalytic intracellular domain. The particular array of sequence motifs of this catalytic intracellular domain determines its access to potential kinase substrates (Mohammadi, et al., 1990, Mol. Cell. Biol. 11:5068-5078; Fantl, et al., 1992, Cell 69:413-413). Examples of receptors that transduce signals via catalytic intracellular domains include the receptor tyrosine kinases (RTKs) such as the Trk family of receptors which are generally limited to cells of the nervous system, the cytokine family of receptors including the tripartate CNTF receptor complex (Stahl & Yancopoulos, 1994, J. Neurobio. 25:1454-1466) which is also generally limited to the cells of the nervous system, G-protein coupled receptors such as the  $\beta_2$ -adrenergic receptor found on, for instance, cardiac muscle cells, and the multimeric IgE high affinity receptor Fc $\epsilon$ RI which is localized, for the most part, on mast cells and basophils (Sutton & Gould, 1993, Nature 366:421-428).

All receptors identified so far appear to undergo dimerization, multimerization, or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci. 13:443-447; Ullrich & Schlessinger, 1990, Cell 61:203-212; Schlessinger & Ullrich, 1992, Neuron 9:383-391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic function. In some instances, such as platelet-derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al., 1988, Science, 240:1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of epidermal growth

factor (EGF), the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem. 259:14631-14636). In the case of the FcεRI receptor, the ligand, IgE, exists bound to FcεRI in a monomeric fashion and only becomes activated when antigen binds to the IgE/FcεRI complex and cross-links adjacent IgE molecules (Sutton & Gould, 1993, Nature 366:421-428).

Often, the tissue distribution of a particular receptor within higher organisms provides insight into the biological function of the receptor. The RTKs for some growth and differentiation factors, such as fibroblast growth factor (FGF), are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, Trends in Cell Biol. 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which bind the Trk RTK family receptors, promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S.E. Loughlin & J.H. Fallon, eds., pp. 257-284, San Diego, CA, Academic Press). FcεRI is localized to a very limited number of types of cells such as mast cells and basophils. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete their maturation in the tissue following migration from the blood stream (See Janeway & Travers, 1996, in Immunobiology, 2d. Edition, M. Robertson & E. Lawrence, eds., pp. 1:3-1:4, Current Biology Ltd., London, UK, Publisher) and are involved in the allergic response.

Many studies have demonstrated that the extracellular domain of a receptor provides the specific ligand binding characteristic.

Furthermore, the cellular environment in which a receptor is expressed may influence the biological response exhibited upon binding of a ligand to the receptor. For example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds to that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, Cell 66:405-413).

10

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF has been purified from conditioned growth media of rat glioma cells [Conn et al., (1990), Proc. Natl. Acad. Sci. U.S.A., 87, pp 2628-2632]; and conditioned growth media of bovine pituitary follicle stellate cells [Ferrara and Henzel, (1989), Biochem. Biophys. Res. Comm., 161, pp. 851-858; Gozpadarowicz et al., (1989), Proc. Natl. Acad. Sci. U.S.A., 86, pp. 7311-7315] and conditioned growth medium from human U937 cells [Connolly, D. T. et al. (1989), Science, 246, pp. 1309-1312]. VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. VEGF has some structural similarities to platelet derived growth factor (PDGF), which is a mitogen for connective tissue cells but not mitogenic for vascular endothelial cells from large vessels.

15

20

25

The membrane-bound tyrosine kinase receptor, known as Flt, was shown to be a VEGF receptor [DeVries, C. et al., (1992), Science, 255, pp.989-

991]. The Flt receptor specifically binds VEGF which induces mitogenesis. Another form of the VEGF receptor, designated KDR, is also known to bind VEGF and induce mitogenesis. The partial cDNA sequence and nearly full length protein sequence of KDR is known as well [Terman, B. I. et al., (1991) Oncogene 6, pp. 1677-1683; Terman, B. I. et al., (1992) Biochem. Biophys. Res. Comm. 187, pp. 1579-1586].

Persistent angiogenesis may cause or exacerbate certain diseases such as psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy and neovascular glaucoma. An inhibitor of VEGF activity would be useful as a treatment for such diseases and other VEGF-induced pathological angiogenesis and vascular permeability conditions, such as tumor vascularization. The present invention relates to a VEGF inhibitor that is based on the VEGF receptor Flt1.

15

Plasma leakage, a key component of inflammation, occurs in a distinct subset of microvessels. In particular, in most organs plasma leakage occurs specifically in the venules. Unlike arterioles and capillaries, venules become leaky in response to numerous inflammatory mediators including histamine, bradykinin, and serotonin. One characteristic of inflammation is the plasma leakage that results from intercellular gaps that form in the endothelium of venules. Most experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of postcapillary and collecting venules (Baluk, P., et al., Am. J. Pathol. 1998 152:1463-76). It has been shown that certain lectins may be used to reveal features of focal sites of plasma leakage, endothelial gaps, and finger-like processes at endothelial cell borders in inflamed venules (Thurston, G., et al., Am. J.

Physiol, 1996, 271: H2547-62). In particular, plant lectins have been used to visualize morphological changes at endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as conconavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond to sites of plasma leakage (Thurston, G., et al., Am J Physiol, 1996, 271: H2547-62).

The properties of the microvessels are dynamic. Chronic inflammatory diseases, for example, are associated with microvascular remodeling, including angiogenesis and microvessel enlargement. Microvessels can also remodel by acquiring abnormal phenotypic properties. In a murine model of chronic airway inflammation, airway capillaries acquire properties of venules, including widened vessel diameter, increased immunoreactivity for von Willebrand factor, and increased immunoreactivity for P-selectin. In addition, these remodeled vessels leak in response to inflammatory mediators, whereas vessels in the same position in the airways of normal mice do not.

Certain substances have been shown to decrease or inhibit vascular permeability and/or plasma leakage. For example, mystixins are synthetic polypeptides that have been reported to inhibit plasma leakage without blocking endothelial gap formation (Baluk, P., et al., J. Pharmacol. Exp. Ther., 1998, 284: 693-9). Also, the beta 2-adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting endothelial gap formation (Baluk, P. and McDonald, D.M., Am. J. Physiol., 1994, 266:L461-8).

The angiopoietins and members of the vascular endothelial growth factor (VEGF) family are the only growth factors thought to be largely specific for vascular endothelial cells. Targeted gene inactivation studies in mice have shown that VEGF is necessary for the early stages of vascular development and that Ang-1 is required for later stages of vascular remodeling.

US Patent No. 6,011,003, issued January 4, 2000, in the name of Metris Therapeutics Limited, discloses an altered, soluble form of FLT polypeptide being capable of binding to VEGF and thereby exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin domains.

US Patent No. 5,712,380, issued January 27, 1998 and assigned to Merck & Co., discloses vascular endothelial cell growth factor (VEGF) inhibitors that are naturally occurring or recombinantly engineered soluble forms with or without a C-terminal transmembrane region of the receptor for VEGF.

Also assigned to Merck & Co. is PCT Publication No. WO 98/13071, published April 2, 1998, which discloses gene therapy methodology for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding a soluble receptor protein which binds to VEGF.

25

PCT Publication No. WO 97/44453, published November 27, 1997, in the name of Genentech, Inc., discloses novel chimeric VEGF receptor proteins comprising amino acid sequences derived from the vascular



endothelial growth factor (VEGF) receptors Flt1 and KDR, including the murine homologue to the human KDR receptor FLK1, wherein said chimeric VEGF receptor proteins bind to VEGF and antagonize the endothelial cell proliferative and angiogenic activity thereof.

5

PCT Publication No. WO 97/13787, published April 17, 1997, in the name of Toa Gosei Co., LTD., discloses a low molecular weight VEGF inhibitor usable in the treatment of diseases accompanied by neovascularization such as solid tumors. A polypeptide containing the first immunoglobulin-like domain and the second immunoglobulin-like domain in the extracellular region of a VEGF receptor FLT but not containing the sixth immunoglobulin-like domain and the seventh immunoglobulin-like domain thereof shows a VEGF inhibitory activity.

10

Sharifi, J. et al., 1998, The Quarterly Jour. of Nucl. Med. 42:242-249, disclose that because monoclonal antibodies (MAbs) are basic, positively charged proteins, and mammalian cells are negatively charged, the electrostatic interactions between the two can create higher levels of background binding resulting in low tumor to normal organ ratios. To overcome this effect, the investigators attempted to improve MAb clearance by using various methods such as secondary agents as well as chemical and charge modifications of the MAb itself.

20

Jensen-Pippo, et al., 1996, Pharmaceutical Research 13:102-107, disclose that pegylation of a therapeutic protein, recombinant human granulocyte colony stimulating factor (PEG-G-CSF), results in an increase in stability and in retention of *in vivo* bioactivity when administered by the intraduodenal route.

25

Tsutsumi, *et al.*, 1997, Thromb Haemost. 77:168-73, disclose experiments wherein the *in vivo* thrombopoietic activity of polyethylene glycol-modified interleukin-6 (MPEG-IL-6), in which 54%  
5 of the 14 lysine amino groups of IL-6 were coupled with PEG, was compared to that of native IL-6.

Yang, *et al.*, 1995, Cancer 76:687-94, disclose that conjugation of polyethylene glycol to recombinant human interleukin-2 (IL-2) results  
10 in a compound, polyethylene glycol-modified IL-2 (PEG-IL-2) that retains the *in vitro* and *in vivo* activity of IL-2, but exhibits a markedly prolonged circulating half-life.

R. Duncan and F. Spreafico, Clin. Pharmacokinet. 27: 290-306, 296  
15 (1994) review efforts to improve the plasma half-life of asparaginase by conjugating polyethylene glycol.

PCT International Publication No. WO 99/03996 published January 28, 1999 in the name of Regeneron Pharmaceuticals, Inc. and The Regents of  
20 The University of California describes modified human noggin polypeptides having deletions of regions of basic amino acids. The modified human noggin polypeptides are described as retaining biological activity while having reduced affinity for heparin and superior pharmacokinetics in animal sera as compared to the  
25 unmodified human noggin.

## SUMMARY OF THE INVENTION

The present invention is directed to VEGF antagonists with improved pharmacokinetic properties. A preferred embodiment is an isolated  
5 nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to (b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion  
10 polypeptide and wherein the nucleotide sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

15

In a further embodiment, the isolated nucleic acid of the first VEGF receptor is Flt1.

20

In a further embodiment, the isolated nucleic acid of the second VEGF receptor is Flk1.

In yet another embodiment, the isolated nucleic acid of the second VEGF receptor is Flt4.

25

In another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain  
5 3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

10 In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule  
15 comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

- (a) the nucleotide sequence set forth in Figure 13A-13D;
- 20 (b) the nucleotide sequence set forth in Figure 14A-14C;
- (c) the nucleotide sequence set forth in Figure 15A-15C;
- (d) the nucleotide sequence set forth in Figure 16A-16D;
- (e) the nucleotide sequence set forth in Figure 21A-21C;
- (f) the nucleotide sequence set forth in Figure 22A-22C;
- 25 (g) the nucleotide sequence set forth in Figure 24A-24C; and
- (h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c ), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having

the biological activity of the modified Flt1 receptor fusion polypeptide.

In a further embodiment of the invention, a fusion polypeptide is encoded by the isolated nucleic acid molecules described above.

5

A preferred embodiment is a composition capable of binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide.

10 Also preferred is a composition wherein the multimer is a dimer.

In yet another embodiment, the composition is in a carrier.

Another embodiment is a vector which comprises the nucleic acid  
15 molecules described above, including an expression vector comprising a the nucleic acid molecules described wherein the nucleic acid molecule is operatively linked to an expression control sequence.

Other included embodiments are a host-vector system for the  
20 production of a fusion polypeptide which comprises the expression vector, in a suitable host cell; the host-vector system wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell; the host-vector system wherein the suitable host cell is E. Coli; the host-vector system wherein the suitable host cell is a  
25 COS cell; the host-vector system wherein the suitable host cell is a CHO cell.

Another embodiment of the invention is a method of producing a fusion polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

5

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D or Figure 24A-24C, which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar

10 excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

15 A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

20

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

A preferred embodiment of the invention is a method of blocking blood  
25 vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

Also preferred is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

- 5 Preferred embodiments of these methods are wherein the mammal is a human.

Further embodiments of the methods of the invention include attenuation or prevention of tumor growth in a human; attenuation or prevention of edema in a human, especially wherein the edema is brain  
10 edema; attenuation or prevention of ascites formation in a human, especially wherein the ascites is ovarian cancer-associated ascites.

Preferred embodiments of the invention include a fusion polypeptide  
15 capable of binding a VEGF polypeptide comprising (a) a VEGF receptor component operatively linked to (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component in the fusion polypeptide and consists essentially of the amino acid sequence of Ig domain 2 of the extracellular domain of a  
20 first VEGF receptor and the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

In a further embodiment of the fusion polypeptide, the first VEGF receptor is Flt1.

25

In yet a further embodiment of the fusion polypeptide, the second VEGF receptor is Flk1.

Still another embodiment of the fusion polypeptide is one in which the second VEGF receptor is Flt4.

Preferred embodiments include a fusion polypeptide wherein amino acid  
5 sequence of Ig domain 2 of the extracellular domain of the first VEGF  
receptor is upstream of the amino acid sequence of Ig domain 3 of the  
extracellular domain of the second VEGF receptor and a fusion  
polypeptide wherein the amino acid sequence of Ig domain 2 of the  
10 extracellular domain of the first VEGF receptor is downstream of the  
amino acid sequence of Ig domain 3 of the extracellular domain of the  
second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing  
component comprises an immunoglobulin domain including an  
15 embodiment wherein the immunoglobulin domain is selected from the  
group consisting of the Fc domain of IgG, the heavy chain of IgG, and the  
light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an  
20 amino acid sequence of a modified Flt1 receptor, wherein the amino  
acid sequence selected from the group consisting of (a) the amino acid  
sequence set forth in Figure 13A-13D; (b) the amino acid sequence set  
forth in Figure 14A-14C; (c) the amino acid sequence set forth in Figure  
15A-15C; (d) the amino acid sequence set forth in Figure 16A-16D; (e)  
25 the amino acid sequence set forth in Figure 21A-21C; (f) the amino acid  
sequence set forth in Figure 22A-22C; and (g) the amino acid sequence  
set forth in Figure 24A-24C.



Another preferred embodiment is a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above.

- 5 An alternative preferred embodiment is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

### **BRIEF DESCRIPTION OF THE FIGURES**

10

**Figure 1.** IEF gel analysis of unmodified and acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pI, whereas acetylated Flt1(1-3)-Fc is able to enter the gel and equilibrate at pI 5.2.

15

**Figure 2.** Binding of unmodified Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc proteins binds extensive to extracellular matrix components in Matrigel®, whereas acetylated Flt1(1-3)-Fc does not bind.

20

**Figure 3.** Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc in a Biacore-based assay. Acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8).

25

Unmodified Flt1(1-3)-Fc (columns 5-6) appears to only partially compete with Biacore chip-bound Flt1(1-3)-Fc for VEGF binding.

However, washing the bound samples with 0.5M NaCl (columns 7-8) results in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein is exhibiting non-specific binding to the chip that can be eliminated by the salt wash.

5

**Figure 4.** Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc to VEGF in an ELISA-based assay. Both pegylated and acetylated Flt1(1-3)-Fc proteins bind to VEGF with affinities approaching that of unmodified Flt1(1-3)-Fc.

10

**Figure 5.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein and the sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The  $C_{max}$  for the different proteins was as follows: Unmodified: 0.06  $\mu$ g/ml - 0.15  $\mu$ g/ml; acetylated: 1.5  $\mu$ g/ml - 4.0  $\mu$ g/ml; and pegylated: approximately 5  $\mu$ g/ml.

**Figure 6A-6B.** IEF gel analysis of unmodified and step-acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its  $>9.3$  pI, whereas most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55 - 8.43, depending on the degree of acetylation.

**Figure 7.** Binding of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins to Matrigel® coated plates. As with the irrelevant control protein, rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold excess samples) does not exhibit any binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. The 10 fold excess sample shows reduced binding, but the degree of acetylation is not enough to completely block binding to extracellular matrix components.

10

**Figure 8.** Binding of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc in a Biacore-based assay. At a sub-stoichiometric ratio (0.5 µg/ml of either unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or step-acetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml, which approximates a 1:1 stoichiometric ratio, the both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely saturate the available VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

15  
20

**Figure 9.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for

25

unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the  $C_{max}$  was as follows: Unmodified Flt1(1-3)-Fc: 0.06 $\mu$ g/ml; 10 fold excess sample: - 0.7 $\mu$ g/ml, 20 fold excess sample - 2 $\mu$ g/ml, 40 fold excess sample - 4 $\mu$ g/ml, 60 fold excess sample - 2 $\mu$ g/ml, 100 fold excess sample - 1 $\mu$ g/ml.

10

**Figure 10A-10D.** Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

**Figure 11.** Schematic diagram of the structure of Flt1.

15

**Figure 12A and 12B.** Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

**Figure 13A-13D.** Nucleic acid and deduced amino acid sequence of Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc.

20

**Figure 14A-14 C.** Nucleic acid and deduced amino acid sequence of Mut2: Flt1(2-3 $_{\Delta B}$ )-Fc.

**Figure 15A-15C.** Nucleic acid and deduced amino acid sequence of Mut3: Flt1(2-3)-Fc.

25

**Figure 16A-16D.** Nucleic acid and deduced amino acid sequence of Mut4: Flt1(1-3<sub>R->N</sub>)-Fc.

**Figure 17.** Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)<sub>R->N</sub> mutant proteins in a Biacore-based assay. At the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3<sub>R->N</sub>)-Fc is somewhat less efficient at blocking binding

20

**Figure 18.** Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc protein binds more weakly still, and the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3<sub>R->N</sub>)-Fc

25

glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

**Figure 19.** Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

**Figure 20.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins. the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc - 0.7μg/ml.

**Figure 21A-21C.** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

**Figure 22A-22C.** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

**Figure 23.** Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

**Figure 24A-24C.** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-Fc $\Delta$ C1(a).

**Figure 25A-25C.** Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.Fc $\Delta$ C1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

**Figure 26A-26B.** Phosphorylation assay. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either transient Flt1D2Flk1D3.Fc $\Delta$ C1(a), stable Flt1D2Flk1D3.Fc $\Delta$ C1(a), or transient VEGFR1R2-Fc $\Delta$ C1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand

during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

5 **Figure 27.** MG/R2 Cell proliferation assay. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined  
10 media was then added to all the wells at a concentration of 1.56nM. The negative control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.Fc $\Delta$ C1(a) blocks 1.56nM VEGF165 with a half maximal dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) are less effective in blocking  
15 VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

**Figure 28.** Biacore analysis of Binding Stoichiometry. Binding  
20 stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) molecule.



**Figure 29 and Figure 30. Size Exclusion Chromatography**

Stoichiometry. Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165

5 interaction) were mixed with varied concentrations of VEGF165. After incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured. The data shows that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3.FcΔC1(a) solution completely blocks Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result  
10 suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule.

**Figure 31. Size Exclusion Chromatography (SEC) under native conditions.** Peak #1 represents the Flt1D2Flk1D3.FcΔC1(a)/ VEGF165  
15 complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl) was added to a final concentration 4.5M to dissociate the complex.

20 **Figure 32. Size Exclusion Chromatography (SEC) under dissociative conditions.** To separate the components of the receptor-ligand complex and to determine their molar ratio, 50μl of dissociated complex was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2  
25 represents VEGF165.

**Figure 33, Figure 34 and Figure 35. Size Exclusion**

Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering

5 detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165.

10 MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak

15 position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

**Figure 36.** Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were

20 determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two

25 cysteines in another Fc chain. However, it can not be determined

whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cys410.

5

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) and are found to be glycosylated to varying degrees. Complete glycosylation is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Asn65 and Asn120. Sites  
10 of glycosylation are highlighted by underline in the Figure.

**Figure 37. Pharmacokinetics of Flt1(1-3)-Fc (A40),**

Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a). Balb/c mice were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO  
15 transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-  
20 FcΔC1(a). The T<sub>max</sub> for Flt1(1-3)-Fc (A40) was at 6 hrs while the T<sub>max</sub> for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The C<sub>max</sub> for Flt1(1-3)-Fc (A40) was 8μg/ml, For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C<sub>max</sub> was 18μg/ml and the C<sub>max</sub> for the  
25 stable VEGFR1R2-FcΔC1(a) was 30μg/ml.

**Figure 38.** Pharmacokinetics of Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). Balb/c mice were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO  
5 transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). Flt1(1-3)-Fc (A40) could no longer be  
10 detected in the serum after day 5 whereas Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more.

**Figure 39.** The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit HT-1080 Fibrosarcoma Tumor Growth In Vivo. Every other day or 2 times per  
15 week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) at 25mg/Kg significantly decreases the growth of subcutaneous HT-1080 fibrosarcoma tumors.

**Figure 40.** The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit C6 Glioma  
20 Tumor Growth In Vivo. Every other day or 2 times a week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) significantly decreases the growth of subcutaneous C6 glioma tumors at doses as low as 2.5mg/Kg.

**Figure 41.** VEGF-Induced Uterine Hyperpermeability. PMSG injected  
25 subcutaneously (5 IU) to induce ovulation in prepubertal female rats

results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. This induction results in hyperpermeability of the uterus and an increase in uterine wet.

Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight.

**Figure 42A-42B.** Assessment of Corpus Luteum Angiogenesis Using Progesterone as a Readout. PMSG was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats, resulting in a fully functioning corpus luteum containing a dense network of blood vessels that secretes progesterone into the blood stream to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF. Resting levels of progesterone are about 5ng/ml and can be induced to 25-40ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG injection resulted in a complete inhibition of the progesterone induction on day 4.

## 20                    DETAILED DESCRIPTION OF THE INVENTION

It has been a long standing problem in the art to produce a receptor based VEGF antagonist that has a pharmacokinetic profile that is appropriate for consideration of the antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric

polypeptide molecule, capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptor-based VEGF antagonists. The chimeric polypeptide molecules described herein thus provide for the first time appropriate  
5 molecules for use in therapies in which antagonism of VEGF is a desired result.

The present invention provides for novel chimeric polypeptide molecules formed by fusing a modified extracellular ligand binding  
10 domain of the Flt1 receptor to the Fc region of IgG.

The extracellular ligand binding domain is defined as the portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can contact with its cognate ligand.  
15 The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar amino acids  
20 (i.e. lysine, arginine, histidine, glutamic acid, aspartic acid). The preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e. leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that precede the hydrophobic transmembrane stretch of amino  
25 acids. Usually the transmembrane domain is flanked by positively

charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains

5 (See von Heijne, 1995, BioEssays 17:25-30). Alternatively, websites on the Internet, such as

[http://ulrec3.unil.ch/software/TMPRED\\_form.html](http://ulrec3.unil.ch/software/TMPRED_form.html). have become available to provide protein chemists with information about making predictions about protein domains.

10

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide molecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells

15 include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control

20 signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination) (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience,

25 NY).

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the chimeric polypeptide molecules include, but are not limited to, the long terminal repeat as described in Squinto et al., (1991, *Cell* 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:144-1445), the regulatory sequences of the metallothioneine gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25, see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and



have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogam et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acid as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF.

Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying

the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

- 5 Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique  
10 which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for  
15 example, Builder, et al., US Patent No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

- 20 In one embodiment of the invention, the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component. In another embodiment of the invention, the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component. Further  
25 embodiments of the invention may be prepared in which the order of the

first, second and third fusion polypeptide components are rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5' to 3' may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or

trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

5

An amino acid sequence analysis of Flt1(1-3)-Fc revealed the presence of an unusually high number (46) of the basic amino acid residue lysine. An IEF analysis of Flt1(1-3)-Fc showed that this protein has pI greater than 9.3, confirming the prediction that the protein is very basic. It was hypothesized that the basic nature of Flt1(1-3)-Fc protein was causing it to bind to extracellular matrix components and that this interaction might be the cause of the extremely short detectable circulating serum half-life exhibited by Flt1(1-3)-Fc when injected into mice. In order to test this hypothesis, Flt1(1-3)-Fc protein was acetylated at the lysine residues to reduce the basic charge. Acetylated Flt1(1-3)-Fc was then tested in the assays described *infra*.

10  
15

The following examples are offered by way of illustration and not by way of limitation.

20

### EXAMPLES

#### Example 1: Expression of Flt1(1-3)-Fc protein in CHO K1 cells.

Using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor

25

Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), the gene encoding Flt1(1-3)-Fc was inserted into the expression vector pEE14.1 (Lonza Biologics, plc) at a multiple cloning site downstream of the CMV promoter. CHO

5 K1 cells were transfected with the pEE14.1/Flt1(1-3)-Fc DNA construct using lipofectamine (Gaithersburg, MD). The transfected CHO K1 cells were grown in glutamine-free DMEM (JRH, Kansas City, MO) containing 25 $\mu$ M methionine sulfoximine (MSX) from Sigma Inc., St. Louis, MO, and high recombinant protein expressors were obtained by

10 screening the CHO K1 cell supernatants from over 100 hand-picked colony isolates using a standard immunoassay which captures and detects human Fc. The selected hand-picked clone was amplified in the presence of 100  $\mu$ M MSX followed by a second round of screening of the amplified clones. The highest producing clone had a specific

15 productivity of recombinant Flt1(1-3)-Fc protein of 55 pg/cell/day.

The selected clone was expanded in 225cm<sup>2</sup> T-flasks (Corning, Acton, MA) and then into 8.5L roller bottles (Corning, Acton, MA) using the cell culture media described *supra*. Cells were removed from the roller

20 bottles by standard trypsinization and put into 3.5L of suspension medium. The suspension medium is comprised of glutamine-free ISCHO medium (Irvine Scientific, Santa Ana, CA) containing 5% fetal bovine serum (FBS from Hyclone Labs, Logan, UT), 100 $\mu$ M MSX and GS supplement (JRH Scientific, Kansas City, MO) in a 5L Celligen

25 bioreactor (New Brunswick Scientific, New Brunswick, NJ) at a density

of  $0.3 \times 10^6$  cells/mL. After the cells reached a density of  $3.6 \times 10^6$ /mL and were adapted to suspension they were transferred to a 60L bioreactor (ABEC, Allentown, PA) at a density of  $0.5 \times 10^6$  cells/mL in 20L of ISCHO medium with 5% fetal bovine serum. After two days an  
5 additional 20L of ISCHO + 5% fetal bovine serum was added to the bioreactor. The cells were allowed to grow for an additional two days reaching a final density of  $3.1 \times 10^6$  cells/mL, and a final Flt1(1-3)-Fc concentration at harvest was 95 mg/L. At harvest the cells were removed by tangential flow filtration using 0.45 $\mu$ m ProstaK Filters  
10 (Millipore, Inc., Bedford, MA).

**Example 2: Purification of Flt1(1-3)-Fc protein obtained from CHO K1 cells**

15 Flt1(1-3)-Fc protein was initially purified by affinity chromatography. A Protein A column was used to bind, with high specificity, the Fc portion of the molecule. This affinity-purified protein was then concentrated and passed over a SEC column. The protein was then eluted into the formulation buffer. The following describes these  
20 procedures in detail.

**Materials and Methods**

All chemicals were obtained from J.T. Baker, Phillipsburg, NJ with the  
25 exception of PBS; which was obtained as a 10X concentrate from Life

Technologies, Gaithersburg, MD. Protein A Fast Flow and Superdex 200 preparation grade resins were obtained from Pharmacia, Piscataway, NJ. Equipment and membranes for protein concentration were obtained from Millipore, Bedford, MA.

5

Approximately 40L of 0.45 $\mu$ m-filtered CHO conditioned media containing Flt1(1-3)-Fc protein was applied to a 290mL Protein A Fast Flow column (10cm diameter) that had been equilibrated with PBS. The column was washed with PBS containing 350mM NaCl and 0.02% CHAPS and the bound protein was eluted with 20mM Citric Acid containing 10mM Na<sub>2</sub>HPO<sub>4</sub>. The single peak in the elution was collected and its pH was raised to neutrality with 1M NaOH. The eluate fractions was concentrated to approximately 9 mg/mL using 10K regenerated cellulose membranes by both tangential flow filtration and by stirred cell concentration. To remove aggregates and other contaminants, the concentrated protein was applied to a column packed with Superdex 200 preparation grade resin (10cm x 55cm) and run in PBS containing 5 % glycerol. The main peak fractions were pooled, sterile filtered, aliquoted and stored at -80°C.

20

**Example 3: Acetylation of Flt1(1-3)-Fc protein.**

Two milligrams of Flt1(1-3)-Fc protein were acetylated as described in the instruction manual provided with the sulfo-NHS-acetate modification kit (Pierce Chemical Co., Rockford, IL, Cat.#26777).

25



**Example 4: Characterization of acetylated Flt1(1-3)-Fc protein.**

5   **(a.) IEF analysis:** Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were analyzed by standard IEF analysis. As shown in Figure 1, Flt1(1-3)-Fc protein is not able to migrate into the gel and therefore must have a pI greater than 9.3, the highest pI in the standard. However, acetylated Flt1(1-3)-Fc is able to migrate into the gel and equilibrate at a pI of  
10 approximately 5.2. This result demonstrates that acetylation reduces the net positive charge of the protein and therefore its pI considerably.

**(b.) Binding to extracellular matrix components**

15 To test for binding to extracellular matrix components, Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were tested in an assay designed to mimic the interaction with extracellular matrix components. In this assay, 96-well tissue culture plates are coated with Matrigel (Biocoat MATRIGEL® matrix thin layer 96 well plate, Catalog #40607, Becton  
20 Dickinson Labware, Bedford, MA). The plates are incubated with varying concentrations of either Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, or rTie2-Fc (an irrelevant control) protein are added to the wells. The plates are incubated for 1-2 hours at either room temperature or 37°C degrees and then detection of bound proteins is accomplished by adding  
25 a secondary alkaline phosphatase-conjugated anti-human Fc antibody to

the wells. Finally, alkaline phosphatase substrate is added to the wells and optical density is measured. Figure 2 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, acetylated Flt1(1-3)-Fc does not exhibit any binding to the Matrigel coated plate, whereas  
5 the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. This result indicates that acetylation of basic amino acid residues is an effective way to interfere with the charge interactions that exist between positively charged proteins and the negatively charged extracellular matrix components they are exposed to *in vivo*.

10

**Example 5: Pegylation of Flt1(1-3)-Fc protein.**

Although pegylation (polyethylene glycol - PEG) of proteins has been shown to increase their *in vivo* potency by enhancing stability and  
15 bioavailability while minimizing immunogenicity (see references cited *supra*), it is counter-intuitive that pegylating molecules that are too large to be filtered by the kidney glomeruli would improve their pharmacokinetic properties. Without being bound by theory, Applicants postulated that pegylation of the Flt1(1-3)-Fc molecules could improve  
20 the pharmacokinetic properties, possibly not by altering the positive charge or by decreasing the pI of Flt1(1-3)-Fc, but rather by physically shielding the positive charges from interacting with the extracellular matrix. Applicants decided to attempt to improve the pharmacokinetic properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs  
25 as described *infra*.

## **Materials and Methods**

Purified Flt1(1-3)-Fc derived from CHO cells (see *supra*) was used in  
5 the following pegylation experiments. Functionalized PEGs were  
obtained from Shearwater Polymers, Huntsville, AL; Bicine from Sigma,  
St Louis, MO; Superose 6 column from Pharmacia, Piscataway, NJ; PBS  
as a 10X concentrate from Life Technologies, Gaithersburg, MD; Glycerol  
from J.T. Baker, Phillipsburg, NJ; and Bis-Tris precast gels from Novex,  
10 CA.

20K PEG strands functionalized with amine-specific terminal moieties  
were used in small-scale reaction studies that were set-up to evaluate  
different reaction conditions in which the PEG:protein stoichiometry  
15 was varied. Based on these reactions and the analyses of samples on  
standard SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was  
reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate)  
molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The  
reaction was allowed to proceed at 8°C overnight. For initial  
20 purification, the reaction products were applied to a 10mm x 30cm  
Superose 6 column equilibrated with PBS containing 5% Glycerol. The  
column appeared to separate pegylated Flt1(1-3)-Fc molecules based on  
the extent of pegylation. Fractions corresponding to what appeared to  
be primarily mono-pegylated and di-pegylated dimeric Flt1(1-3)-Fc, as  
25 judged by banding patterns on reducing and non-reducing SDS-PAGE gels

were pooled. The protein concentration was determined by measuring absorbance at 280 nm. The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at -40°C.

**5 Example 6: Binding of unmodified, acetylated, and pegylated Flt1(1-3)-Fc in a Biacore-based assay.**

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a Biacore-based assay to evaluate their ability to bind to the  
10 Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard procedures) and a sample containing 0.2 µg/ml VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or pegylated Flt1(1-3)-Fc (each at 25 µg/ml)  
15 was passed over the Flt1(1-3)-Fc-coated chip. To minimize the effects of non-specific binding, the bound samples were washed with a 0.5M NaCl wash. In one sample, unmodified Flt1(1-3)-Fc was mixed with heparin. Heparin is a negatively charged molecule and the Flt1(1-3)-Fc protein is a positively charged molecule, so when the two molecules  
20 are mixed together, they should interact through their respective charges. This essentially neutralizes Flt1(1-3)-Fc's inherent positive charge making the molecule behave as if it has been chemically or genetically modified so as to reduce its charge and its tendency to bind via charge interactions. As shown in Figure 3, acetylated (columns 13-  
25 16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc

(columns 21-24) are each able to completely compete with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appeared to only partially compete with Biacore chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5M NaCl (columns 7-8) resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

10

**Example 7: Binding of unmodified, acetylated, and pegylated Flt1(1-3)-Fc in an ELISA-based assay.**

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a standard ELISA-based assay to evaluate their ability to bind the Flt1 receptor ligand VEGF. As shown in Figure 4, both pegylated and acetylated Flt1(1-3)-Fc proteins are capable of binding to VEGF, demonstrating that modifying the protein either by pegylation or acetylation does not destroy its ability to bind its ligand.

20

**Example 8: Pharmacokinetic analysis of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc.**

*In vivo* experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and

pegylated Flt1(1-3)-Fc protein. Balb/c mice (23-28g; 3 mice/group) were injected subcutaneously with 4mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein. The sera were  
5 assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acetylated, or pegylated Flt1(1-3)-Fc-containing sera, and reporting with an anti-Fc antibody linked to alkaline phosphatase. As shown in Figure 5, the Tmax for all of the  
10 Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The Cmax for the different proteins was as follows: Unmodified: 0.06  $\mu$ /ml - 0.15  $\mu$ g/ml; acetylated: 1.5  $\mu$ g/ml - 4.0  $\mu$ g/ml; and pegylated: approximately 5  $\mu$ g/ml.

### 15 **Example 9: Step-acetylation of Flt1(1-3)-Fc**

To determine what minimal amount of acetylation is necessary to eliminate binding to extracellular matrix components, an experiment was designed that acetylated the Flt1(1-3)-Fc protein in a step-wise  
20 fashion by using increasing amounts of molar excess of acetylation reagent in the acetylation reaction mixture. The range of molar excess was as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 moles of acetylation reagent per 1 mole of Flt1(1-3)-Fc monomer. The reactions were performed as detailed in the instruction manual provided with the

sulfo-NHS-Acetate modification kit (Pierce Chemical Co., Rockford, IL, Cat.# 26777).

**Example 10: Characterization of step-acetylated Flt1(1-3)-**

5 **Fc.**

**(a.) IEF analysis** Unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins were analyzed by standard IEF analysis. As shown in Figure 6A-6B, unmodified Flt1(1-3)-Fc protein was not able  
10 to migrate into the gel due to its extremely high pI (greater than 9.3). However, most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold molar excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55 - 8.43, depending on the degree of acetylation of the protein. This result demonstrates that  
15 acetylation can change the positive charge of the protein in a dose-dependent manner and that reduction of the pI can be controlled by controlling the degree of acetylation.

**(b.) Binding of step-acetylated Flt1(1-3)-Fc to extracellular**  
20 **matrix components**

To test for binding to extracellular matrix components, Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc were tested in the above-described assay designed to mimic the interaction with extracellular matrix  
25 components. Varying concentrations of either unmodified Flt1(1-3)-Fc,

step-acetylated Flt1(1-3)-Fc (10, 20, and 30 fold molar excess samples), or rTie2-Fc (an irrelevant control) protein were added to the wells. The plates were incubated for 1-2 hours at room temperature or 37°C and then detection of bound proteins was accomplished by adding a  
5 secondary alkaline phosphatase-conjugated anti-human Fc antibody to the wells. Alkaline phosphatase substrate was subsequently added to the wells and optical density measured. Figure 7 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold molar excess samples) did not  
10 exhibit any significant binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibited significant binding. The binding is saturable, indicating that the Flt1(1-3)-Fc protein may be binding to specific sites, rather than a more general charge-mediated interaction that might not be saturable. The 10 fold molar  
15 excess sample showed reduced binding, but the degree of acetylation was not enough to completely block binding to extracellular matrix components. The 20 fold molar excess and higher samples displayed no detectable binding, despite the fact that by IEF analysis (Figure 6A and 6B) the lower molar excess samples still had a large net positive  
20 charge. This result demonstrates that it is not necessary to completely acetylate all available basic amino acids in order to eliminate binding to extracellular matrix components.



**(c.) Binding of step-acetylated Flt1(1-3)-Fc in a Biacore-based assay.**

Unmodified and step-acetylated Flt1(1-3)-Fc proteins were tested in  
5 a Biacore-based assay to evaluate their ability to bind to the Flt1  
ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.5, 1.0,  
or 5.0 µg/ml) was immobilized on the surface of a Biacore chip (see  
Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for  
standard procedures) and a solution containing 0.2 µg/ml VEGF and  
10 either unmodified Flt1(1-3)-Fc (at either 0.5, 1.0, or 5.0 µg/ml) or 10  
different step-acetylated Flt1(1-3)-Fc samples (at 0.5, 1.0, or 5.0  
µg/ml each) were passed over the Flt1(1-3)-Fc-coated chip. As shown  
in Figure 8, at a sub-stoichiometric ratio (0.5 µg/ml of either  
unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml  
15 VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or step-  
acetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml,  
which approximates a 1:1 stoichiometric ratio, both unmodified and  
step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF  
binding, but there is still insufficient Flt1(1-3)-Fc protein (either  
20 unmodified or step-acetylated) to completely bind the available VEGF.  
However, at 5.0 µg/ml, which is several times greater than a 1:1  
stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated  
Flt1(1-3)-Fc proteins are able to bind the VEGF, regardless of the  
degree of acetylation. This clearly demonstrates that acetylation does  
25 not alter Flt1(1-3)-Fc's ability to bind VEGF.

**(d.) Pharmacokinetic analysis of step-acetylated Flt1(1-3)-Fc**

*In vivo* experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc protein. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc (described *supra*). Figure 9 details the results of this study. The T<sub>max</sub> for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the C<sub>max</sub> was as follows: Unmodified Flt1(1-3)-Fc: 0.06µg/ml; 10 fold molar excess sample: - 0.7µg/ml, 20 fold molar excess sample - 2µg/ml, 40 fold molar excess sample - 4µg/ml, 60 fold molar excess sample - 2µg/ml, 100 fold molar excess sample - 1µg/ml. This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

**Example 11: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut1: Flt1(1-3)<sub>ΔB</sub>-Fc.**

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI

below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc ( $pI > 9.3$ ), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues. Sometimes these residues are found in a cluster on the primary sequence of the protein; some of the literature has identified "consensus sequences" for such heparin binding sites (see for example Hileman, et al., 1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that the residues are spatially close to one another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876). Referring to the nucleic acid and amino acid sequence set forth in Figure 10A-10D

of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys, starting at nucleotide 79 of the nucleic acid  
5 sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly,  
10 encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that  
15 there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of Figure 10A-10D, in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the  
20 sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic  
25 region in the protein (Figure 12A-12B). These observations raised the

possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc. The Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in Figure 10A-10D), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc is set forth in Figure 13A-13D.

**Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc.**

A second deletion mutant construct, designated Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, was derived from the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc is set forth in Figure 14A-14C.

**Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.**

A third deletion mutant construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino

acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C.

5

**Example 14: Construction of Flt1(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3<sub>R->N</sub>)-Fc.**

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3<sub>R->N</sub>)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3<sub>R->N</sub>)-Fc is set forth in Figure 16A-16D.

**Example 15: Characterization of acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, and Mut4: Flt1(1-3<sub>R->N</sub>)-Fc mutants.**

**(a.) Binding to extracellular matrix components**

To determine whether the three modified proteins were more or less

likely to have improved pharmacokinetic properties, Matrigel coated 96-well dishes (as described *supra*) were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkaline-phosphatase conjugated antibodies. As shown in Figure 18, this experiment showed that while the unmodified Flt1(1-3)-Fc protein could bind avidly to these wells, the Mut3: Flt1(2-3)-Fc protein bound somewhat more weakly, the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc protein bound more weakly still, and the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3<sub>R->N</sub>)-Fc glycosylation mutant protein showed only marginal benefit on the Matrigel assay. These results confirm the hypothesis that a linear sequence of positive amino acids can be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

**(b.) Binding of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc and Mut4: Flt1(1-3<sub>R->N</sub>)-Fc in a Biacore-based assay.**

Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc and Mut4: Flt1(1-3<sub>R->N</sub>)-Fc proteins were tested in a Biacore-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.25, 0.5, or 1.0 μg/ml) was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard procedures) and a solution containing 0.1 μg/ml VEGF and either



purified or COS cell supernatant containing unmodified Flt1(1-3)-Fc (at approximately (0.25, 0.5, or 1.0  $\mu\text{g/ml}$ ), purified acetylated Flt1(1-3)-Fc (at (0.25, 0.5, or 1.0  $\mu\text{g/ml}$ ), COS cell supernatant containing Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc (at approximately (0.25, 0.5, or 1.0  $\mu\text{g/ml}$ ), or COS cell  
5 supernatant containing Mut4: Flt1(1-3 $_{R \rightarrow N}$ )-Fc (at approximately (0.25, 0.5, or 1.0  $\mu\text{g/ml}$ ) were passed over the Flt1(1-3)-Fc-coated chip. As shown in Figure 17, at the sub-stoichiometric ratio (0.25  $\mu\text{g/ml}$  Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01.  $\mu\text{g/ml}$  VEGF), there is insufficient Flt1(1-3)-Fc protein to block  
10 binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5  $\mu\text{g/ml}$  of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0  $\mu\text{g/ml}$  of unmodified, acetylated or genetically modified Flt1(1-3)-Fc  
15 proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3 $_{R \rightarrow N}$ )-Fc is somewhat less efficient at  
20 blocking binding. These results confirm the hypothesis that it is possible to reduce the non-specific binding of a positively charged molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

**(c.) Binding of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, Mut3: Flt1(2-3)-Fc, and in an ELISA-based assay.**

To determine whether the three mutant proteins could bind the Flt1  
5 ligand VEGF, binding experiments were done in which 96-well plates  
coated with VEGF were incubated with varying concentrations of the  
respective mutant protein, and after washing, the amount bound was  
detected by incubating with an alkaline phosphatase conjugated anti-  
human Fc antibody and quantitated colorimetrically by the addition of  
10 an appropriate alkaline phosphatase substrate. As shown in Figure 19,  
this experiment showed that all the mutant proteins could bind VEGF  
similarly, at the concentrations tested.

**Example 16: Pharmacokinetic analysis of acetylated Flt1(1-  
15 3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, and unmodified Flt1(1-3)-Fc.**

*In vivo* experiments were designed to assess the pharmacokinetic  
profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, and 40 fold  
molar excess acetylated Flt1(1-3)-Fc protein. Balb/c mice (25-30g)  
20 were injected subcutaneously with 4mg/kg of unmodified Flt1(1-3)-Fc,  
40 fold molar excess acetylated Flt1(1-3)-Fc, and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc  
proteins (4 mice each). These mice were tail bled at 1, 2, 4, 6, 24  
hours, 2 days, 3 days, and 5 days after injection. The sera were  
assayed in an ELISA designed to detect Flt1(1-3)-Fc protein which  
25 involves coating an ELISA plate with VEGF, binding the Flt1(1-3)-Fc and

reporting with an anti-Fc antibody linked to alkaline phosphatase. As shown in Figure 20, the Cmax for these reagents was as follows:

Unmodified Flt1(1-3)-Fc - 0.15µg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5µg/ml; and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc - 0.7µg/ml.

5

**Example 17: Modified Flt1 receptor vector construction**

The rationale for constructing modified versions of the Flt1 receptor (also known as VEGFR1) was based on the observation that the protein  
10 sequence of Flt1 was highly basic, and was therefore likely to stick to extracellular matrix (ECM). The highly basic nature of Flt1 probably explains why unmodified Flt1(1-3)-Fc (described *supra*) has poor pharmacokinetics that make it difficult to use as a therapeutic agent. As described *supra*, the chemically modified form of 40 fold molar  
15 excess acetylated Flt1(1-3)-Fc, hereinafter termed A40, exhibited a greatly improved pharmacokinetic (PK) profile over the non-acetylated Flt1(1-3)-Fc. Therefore, attempts were made to engineer DNA molecules that could be used to recombinantly express modified forms of a Flt1 receptor molecule that would possess the improved PK profile  
20 exhibited by A40 and still maintain the ability to bind tightly to VEGF.

It is known in the literature that the first Ig domain of Flt1 (which has a net charge of +5 at neutral pH) is not essential for tight binding to VEGF, so this domain was deleted. The third Ig domain (having a net  
25 charge of +11) is not essential for binding, but confers higher affinity

for VEGF than the second Ig domain, so instead of deleting it entirely, it was replaced with the equivalent domains of the Flt1 receptor relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3). These chimeric molecules (denoted R1R2 (Flt1.D2.FlklD3.FcΔC1(a) and  
5 VEGFR1R2-FcΔC1(a) and R1R3 (Flt1D2.VEGFR3D3-FcΔC1(a) and VEGFR1R3-FcΔC1(a) respectively, wherein R1 and Flt1D2 = Ig domain 2 of Flt1 (VEGFR1); R2 and FlklD3 = Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3 = Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an *in vitro* ECM binding assay as described *infra*, had  
10 greatly improved PK as described *infra*. In addition, these molecules were able to bind VEGF tightly as described *infra* and block phosphorylation of the native Flk1 receptor expressed in endothelial cells as described *infra*.

15 **(a) Construction of the expression plasmid**  
**pFlt1D2.FlklD3.FcΔC1(a)**

Expression plasmids pMT21.Flt1(1-3).Fc (6519bp) and pMT21.Flkl-1(1-3).Fc (5230bp) are plasmids that encode ampicillin resistance and Fc-  
20 tagged versions of Ig domains 1-3 of human Flt1 and human Flkl1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flkl1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a

single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTTCGTAGAGATG-3')

5

3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3')

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence  
10 GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C). The  
3' primer encodes the reverse complement of the 3' end of Flt1 Ig  
domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with  
the fusion point defined as TIID of Flt1 (corresponding to amino acids  
123-126 of Figure 21A-21C) and continuing into VVLS (corresponding  
15 to amino acids 127-130 of Figure 21A-21C) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:

20 5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG  
G-3')

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC  
AAATG-3')

25

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (corresponding to amino acids 223-228 of Figure 21A-21C), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ $\Delta$ B2.Fc, to create the plasmid pMT21/Flt1D2.FlklD3.Fc. The nucleotide sequence of the Flt1D2-FlklD3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-Fc $\Delta$ C1(a) to produce the plasmid pFlt1D2.FlklD3.Fc $\Delta$ C1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.FlklD3.Fc $\Delta$ C1(a) chimeric molecule is set forth in Figure 21A-21C.

**(b) Construction of the expression plasmid****pFlt1D2VEGFR3D3Fc $\Delta$ C1(a)**

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin  
5 resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1  
receptor. This plasmid was used to produce a DNA fragment containing  
Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used  
to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A  
further round of PCR amplification was used to achieve fusion of the  
10 two Ig domains into a single fused fragment. For Ig domain 2 of Flt1,  
the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

15 3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA  
TTGGT)

The 5' amplification primer encodes a BspE1 restriction site upstream  
of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM  
20 (corresponding to amino acids 27-33 of Figure 22A-22C). The 3'  
amplification primer encodes the reverse complement of the end of Flt1  
Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with  
the fusion point defined as TIID of Flt1 (corresponding to amino acids  
123-126 of Figure 22A-22C) and continuing into IQLL of VEGFR3  
25 (corresponding to amino acids 127-130 of Figure 22A-22C).

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows:

5 5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3.

10 The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

15

5': Flt1D2.VEGFR3D3.s

(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as

20 (GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of

25 VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding



to amino acids 221-226 of Figure 22A-22C), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C.

5

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and

10 VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1 $\Delta$ B2.Fc (described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc.

15 The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3) $\Delta$ B2-Fc $\Delta$ C1(a) to produce the plasmid designated  
20 pFlt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) chimeric molecule is set forth in Figure 22A-22C.

**Example 18: Extracellular Matrix Binding (ECM) Binding Assay**

ECM-coated plates (Becton Dickinson catalog # 35-4607) were rehydrated with warm DME supplemented with glutamine (2mM), 100U penicillin, 100U streptomycin, and 10% BCS for at least 1 hr. before adding samples. The plates were then incubated for 1 hr. at room temperature with varying concentrations of Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) starting at 10nM with subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 hr. at room temperature. The plates were then washed 4 times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP solution (Sigma) was added for color development. Plates were read at  $\lambda = 405-570\text{nm}$ . The results of this experiment are shown in Figure 23 and demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

**Example 19: Transient expression of pFlt1D2.Flk1D3.FcΔC1(a) in CHO-K1 (E1A) cells**

A large scale (2L) culture of E. coli DH10B cells carrying the pFlt1D2.Flk1D3.FcΔC1(a) plasmid described *supra* in Example 17(a) was grown overnight in Terrific Broth (TB) plus 100μg/ml ampicillin. The

next day, the plasmid DNA was extracted using a QIAgen Endofree Megaprep kit following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined by standard techniques using a UV spectrophotometer and fluorometer. The plasmid DNA was

5 verified by standard restriction enzyme digestion of aliquots using the restriction enzymes EcoRI plus NotI and AseI. All restriction enzyme digest fragments corresponded to the predicted sizes when analyzed on a 1% agarose gel.

10 Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of  $4 \times 10^6$  cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% Hyclone Fetal Bovine Serum (FBS), 100U penicillin/100U streptomycin and glutamine (2mM). The following day each plate of cells was transfected with 6  $\mu$ g of the

15 pFlt1D2.Flk1D3.Fc $\Delta$ C1(a) plasmid DNA using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells, 12 ml/plate of Optimem supplemented with 10% FBS was added. Plates were incubated at 37°C in a 5% CO<sub>2</sub> incubator overnight. The following day

20 the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2mM) and 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation, the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet

cells. The supernatant was decanted into sterile 1L bottles and purification of the expressed protein was performed as described *infra*.

**Example 20: Construction pVEGFR1R2-Fc $\Delta$ C1(a) expression**

5 **vector**

The pVEGFR1R2.Fc $\Delta$ C1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Figure 24A-24C) between Flt1d2-Flk1d3-Fc $\Delta$ C1(a) amino acids 26 and 27 of Figure 21A-21C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.Fc $\Delta$ C1(a) chimeric molecule is set forth in Figure 24A-24C.

**Example 21: Cell Culture Process Used to Produce Modified**

20 **Flt1 Receptors**

**(a) Cell Culture Process Used to Produce**

**Flt1D2.Flk1D3.Fc $\Delta$ C1(a)**

25 The process for production of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein using the

expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described *supra* in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography. The process is provided in greater detail below.

### **Cell Expansion**

Two confluent T-225 cm<sup>2</sup> flasks containing the Flt1D2.Flk1D3.FcΔC1(a) expressing cell line were expanded by passaging cells into eight T-225 cm<sup>2</sup> flasks in medium (GMEM + 10% serum, GIBCO) and incubated at 37°C and 5% CO<sub>2</sub>. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm<sup>2</sup> roller bottles and incubated at 37°C and 5% CO<sub>2</sub> until confluent.

### **Suspension Culture in Bioreactors**

Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5L of suspension culture. The

suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25  $\mu$ M methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37°C. When a density of  $4 \times 10^6$  cells/mL was reached the cells were transferred to a 40L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34°C to slow cell growth and increase the relative rate of protein expression.

15 **(b) Cell Culture Process Used to Produce**  
**Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a)**

The same methodologies as described *supra* for Flt1D2.FlklD3.Fc $\Delta$ C1(a) were used to produce Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a).

20 **Example 22: Harvest and Purification of Modified Flt1**  
**Receptors**

**(a) Harvest and Purification of Flt1D2.Flk1D3.FcΔC1(a)**

The product protein was aseptically harvested from the bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40L of harvest filtrate was then loaded onto a 400 mL column containing Protein A Sepharose resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2.Flk1D3.FcΔC1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at -20°C.

Several frozen lots of Flt1D2.Flk1D3.FcΔC1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30kD nominal molecular weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.FcΔC1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

**(b) Harvest and Purification of Flt1D2.VEGFR3D3.FcΔC1(a)**

The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a)  
5 were used to harvest and purify Flt1D2.VEGFR3D3.FcΔC1(a).

**Example 23: Phosphorylation Assay for Transiently Expressed VEGFR2**

10 Primary human umbilical vein endothelial cells (HUVECs), passage 4-6, were starved for 2 hrs in serum-free DME high glucose media. Samples containing 40 ng/ml (1nM) human VEGF165, which is a ligand for the VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and were preincubated for 1 hr. at room temperature with varying amounts of the  
15 modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), Flt1D2Flk1D3.FcΔC1(a) and Flt1D2VEGFR3D3.FcΔC1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for 5 minutes with the samples prepared above +/- VEGF165, followed by whole cell lysis using complete lysis buffer. Cell lysates were  
20 immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane using standard transfer methodologies. Detection of phosphorylated VEGFR2 was done by immunoblotting with the anti-phospho Tyrosine  
25 mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham).



Figures 25A-25C and 26A-26B show the results of this experiment.

Figure 25A-25C reveals that detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1 receptor is used during the preincubations with VEGF. As is seen in Figure 25A, at a 1.5 molar excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.Fc $\Delta$ C1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

In Figure 26A-26B, detection by Western blot of tyrosine

phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either transient Flt1D2Flk1D3.Fc $\Delta$ C1(a), stable Flt1D2Flk1D3.Fc $\Delta$ C1(a), or transient VEGFR1R2-Fc $\Delta$ C1(a). At all modified Flt1 receptor

concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

5

#### **Example 24: Cell Proliferation Bioassay**

The test cell population is MG87 cells that have been stably transfected with a expression plasmid that contains a DNA insert encoding the  
10 VEGFR2(Flk1) extracellular domain fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase domain was used rather than the native VEGFR2(Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2(Flk1) does not cause a strong proliferative  
15 response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full length TrkB receptor give a robust proliferative response when stimulated with BDNF, so the TrkB intracellular kinase domain was engineered to replace the intracellular kinase domain of VEGFR2(Flk1) to take advantage of this proliferative  
20 response capability.

$5 \times 10^3$  cells/well were plated in a 96 well plate and allowed to settle for 2 hrs at 37°C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.FlklD3.Fc $\Delta$ C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a), plus an  
25 irrelevant receptor termed Tie2-Fc as a negative control, were titrated

from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56nM. The plates were incubated for 72 hrs at 37°C and then MTS (Owen's reagent, Promega) added and the plates were  
5 incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570nm. The results of this experiment are shown in Figure 27. The control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56nM VEGF165 with a half maximal  
10 dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

15 **Example 25: Binding Stoichiometry of Modified Flt Receptors to VEGF165**

**(a) BIAcore Analysis**

20 The stoichiometry of Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.FcΔC1(a) or  
25 VEGFR1R2-FcΔC1(a) to VEGF BIAcore chip surface.

Modified Flt receptors Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a), were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip (BIAcore) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over the Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) surfaces at 10 μl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule (Figure 28).

In solution, Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.FcΔC1(a) BIAcore binding signal to its molar concentration. The data showed that the addition of 1 nM VEGF165 into

the Flt1D2Flk1D3.FcΔC1(a) solution completely blocked Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule (Figure 29 and Figure 30). When the concentration of Flt1D2Flk1D3.FcΔC1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.FcΔC1(a) and -1.07 for VEGFR1R2-FcΔC1(a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a).

#### **(b) Size Exclusion Chromatography**

Flt1D2Flk1D3.FcΔC1(a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6M guanidine hydrochloride in order to dissociate it into its component proteins.

Flt1D2Flk1D3.FcΔC1(a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.FcΔC1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under condition identical to one used in separating components of Flt1D2Flk1D3.FcΔC1(a)/VEGF

complex. Quantification of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex composition was based on the calibration curves. The results of this experiment are set forth in Figure 28, which shows the ratio of VEGF165 to Flt1D2Flk1D3.FcΔC1(a) in a complex to be 1:1.

5

**Example 26: Determination of the Binding Stoichiometry of Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography**

10 **Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex Preparation**

VEGF165 (concentration = 3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1D2.Flk1D3.FcΔC1(a) (concentration = 0.9 mg/ml) in molar ratio of 3:1 (VEGF165:Flt1D2.Flk1D3.FcΔC1(a)) and  
15 incubated overnight at 4°C.

**(a) Size Exclusion Chromatography (SEC) under native conditions**

20 To separate the complex from excess of unbound VEGF165, 50 μl of the complex was loaded on a Pharmacia Superose 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40μl/min. at room temperature. The results of this SEC are shown in Figure 31. Peak #1 represents the complex and peak  
25 #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2

ml were combined and guanidinium hydrochloride (GuHCl) was added to a final concentration 4.5M to dissociate the complex.

**(b) Size Exclusion Chromatography (SEC) under dissociative conditions**

To separate the components of the receptor-ligand complex and to determine their molar ratio, 50 $\mu$ l of dissociated complex as described *supra* was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted with the same solution at a flow rate 40 $\mu$ l/min. at room temperature. The results of this SEC are shown in Figure 32. Peak #1 represents Flt1D2Flk1D3.Fc $\Delta$ C1(a) and peak #2 represents VEGF165.

**(c ) Calculation of Flt1D2Flk1D3.Fc $\Delta$ C1(a):VEGF165 Complex Stoichiometry**

The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the components. Concentrations of VEGF165 and Flt1D2Flk1D3.Fc $\Delta$ C1(a) corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165 and Flt1D2Flk1D3.Fc $\Delta$ C1(a). To obtain a standard curve, four different concentrations (0.04 mg/ml -0.3mg/ml) of either component were injected onto a Pharmacia Superose 12 PC 3.2/30 column equilibrated in 6M guanidinium chloride and eluted with the same

solution at flow rate 40 $\mu$ l/min. at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165:Flt1D2Flk1D3.Fc $\Delta$ C1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165:Flt1D2Flk1D3.Fc $\Delta$ C1(a) determined from the peak height of the components was 1.10.

**Example 27: Determination of the Stoichiometry of the Flt1D2Flk1D3.Fc $\Delta$ C1(a)/VEGF165 Complex by Size Exclusion**

**Chromatography with On-Line Light Scattering**

**Complex preparation**

VEGF165 was mixed with CHO transiently expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3.Fc $\Delta$ C1(a)) and incubated overnight at 4°C.

**(a) Size Exclusion Chromatography (SEC) with On-Line Light Scattering**

20

Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex.

25 Samples were injected onto a Superose 12 HR 10/30 column



(Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex is 1:1 as it corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

## **Example 28: Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)**

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass

spectrometry, in addition to an N-terminal sequencing technique.

Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify

5 fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36.

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide  
10 bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined  
15 whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

20 There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is

observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36.

**Example 29: Pharmacokinetic Analysis of Modified Flt**

**5 Receptors**

**(a) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40).**

**Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)**

- 10 Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24hrs, 2 days, 3 days and 6 days after injection. The sera were assayed
- 15 in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results
- 20 of this experiments are shown in Figure 37. The  $T_{max}$  for Flt1(1-3)-Fc (A40) was at 6 hrs while the  $T_{max}$  for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The  $C_{max}$  for Flt1(1-3)-Fc (A40) was 8μg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the  $C_{max}$

was 18µg/ml and the  $C_{\max}$  for the stable VEGFR1R2-FcΔC1(a) was 30µg/ml.

**(b) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40).**

**5 Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a)**

Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice  
10 were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting  
15 with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas , Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more. The results of this experiment are shown in Figure 38.

20

**Example 30: Evaluation of the Ability of**  
**Flt1D2.Flk1D3.FcΔC1(a) to Inhibit Tumor Growth In Vivo**

To evaluate the ability of Flt1D2.Flk1D3.FcΔC1(a) to inhibit tumor  
25 growth in vivo a model in which tumor cell suspensions are implanted

subcutaneously on the right flank of male severe combined immunodeficiency (SCID) mice was employed. Two cell lines, the human HT-1080 fibrosarcoma cell line (ATCC accession no. CCL-121) and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of which exhibit distinctly different morphologies and growth characteristics, were used in the assay. The first dose of Flt1D2.Flk1D3.FcΔC1(a) (at 25mg/Kg or as indicated in Figures 39 and 40) was given on the day of tumor implantation. Animals subsequently received subcutaneous injections of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or vehicle either every other day (EOD) or two times per week (2X/wk) for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and Flt1D2.Flk1D3.FcΔC1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells. The results of these experiments are shown in Figure 39 and Figure 40.

### **Example 31: The Effect of VEGF165 and Modified Flt**

#### **Receptors in Female Reproductive System**

The stereotypic pattern of vascular remodeling which occur in the uterus and ovary over the course of the reproductive cycle has been well characterized, making these tissues particularly well suited to the study of mechanisms which regulate angiogenesis, vascular

remodeling and vascular regression. Indeed, *in situ* hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates (Phillips et al, 5 1990; Ravindranath et al, 1992; Shweiki et al, 1993; Kamat et al, 1995). As cyclic angiogenesis and vascular remodeling are prominent features of the normal ovary and uterus, it is not surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these 10 organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or anti-angiogenic factors, most prominently VEGF.

15 For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the affected tissue (Kamat et al, 1995; Shifren et al, 1996; Guidi et al, 1996; Donnez et al, 1998).

Overexpression of VEGF is also thought to play a pathogenic role in the 20 establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome (McClure et al, 1994; Levin et al, 1998) and preeclampsia (Baker et al, 1995; Sharkey et al, 1996). In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other 25 tumors (Senger et al, 1983; Boock et al, 1995). Agents which effectively neutralize the biological actions of VEGF can reasonably be

anticipated to be of therapeutic benefit in the above and related conditions.

Angiogenesis and vascular remodeling are also hallmarks of blastocyst  
5 implantation and placental development (Findlay, 1986). VEGF is  
strongly expressed both in the maternal decidua and in embryonic  
trophoblasts, where it is thought to first stimulate expansion and  
hyperpermeability of the uterine vasculature during the peri-  
10 implantation period and subsequently mediate formation of both the  
maternal and embryonic components of the placental vasculature  
(Shweiki et al, 1993; Cullinan-Bove and Koos, 1993; Chakraborty et al,  
1995; Das et al, 1997). VEGF is also required for luteal angiogenesis  
and associated progesterone secretion necessary to prepare the uterus  
15 for implantation (Ferrara et al, 1998). Thus, agents which inhibit the  
biological actions of VEGF may prove to be useful as contraceptive  
agents (by preventing implantation), or as abortifacients in the early  
stages of gestation. The latter application might find particular use as  
a non-surgical intervention for the termination of ectopic pregnancies.

20 While the expression of VEGF receptors is largely confined to the  
vascular endothelium in normal reproductive tissues, Flt1 is also  
expressed by trophoblasts in the placenta in both humans and animals  
(Clark et al, 1996; He et al, 1999) where it has been proposed to play a  
role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are  
25 expressed by choriocarcinoma cell line BeWo (Charnock-Jones et al,  
1994), and VEGF has been shown to promote DNA synthesis and tyrosine  
phosphorylation of MAP kinase in these cells. Furthermore, primary and

metastatic ovarian carcinomas not only to express high levels of VEGF, but - in addition to the vascular endothelium - the tumor cells themselves express KDR and/ or Flt1 (Boocock et al, 1995). These findings suggest that VEGF may not only be critically involved in the generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

## **Methods and Results**

### **(a) Assessment of VEGF-Induced Uterine Hyperpermeability**

Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by the modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). In this in vivo model, the normal weight of the rat uterus is about 50 mg and this can be induced to 300-350 mg by PMSG. Desiccation of the tissue reveals that this is all water weight. Subcutaneous injection of Flt1(1-3)-Fc (A40),



Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr. after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight. Increasing the dose of modified Flt receptor does not further reduce the increase in wet weight suggesting  
5 that there is a VEGF-independent component to this model. The results of this experiment are shown in Figure 41.

**(a) Assessment of corpus luteum angiogenesis using progesterone as a readout**

10

Pregnant mare's serum gonadotrophin (PMSG) is injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a fully functioning corpus luteum containing a dense network of blood vessels after 4 days that allows for the secretion of  
15 progesterone into the blood stream in order to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF; therefore, blocking VEGF would result in a lack of new blood vessels and thus a lack of progesterone secreted into the blood stream. In this in vivo model, resting levels of progesterone are about  
20 5ng/ml and this can be induced to a level of 25-40ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG injection results in a complete inhibition of the progesterone induction on day 4. The results of this experiment are shown in Figure 42A-42B.

25

**Example 33: Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc**

Flt1(1-3)-Fc was PEGylated with either 10kD PEG or 20kD PEG and tested in balb/c mice for their pharmacokinetic profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the T<sub>max</sub> occurring at 24 hrs. for the PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

**Example 34: VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants**

10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3<sub>NAS</sub>)-Fc, Flt1(1-3<sub>R->C</sub>)-Fc and Tie2-Fc. Flt1(1-3<sub>NAS</sub>)-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR is replaced by NASVNGSR, resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-3<sub>R->C</sub>)-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR ->

KNKCASVRRR) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK.

After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was

5 then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-3<sub>R→C</sub>)-Fc, Flt1(1-3<sub>NAS</sub>)-Fc and

10 Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

WE CLAIM:

1. An isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising:

5 (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to

(b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide and wherein the nucleotide

10 sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

15

2. The isolated nucleic acid of claim 1 wherein the first VEGF receptor is Flt1.

3. The isolated nucleic acid of claim 1 wherein the second VEGF  
20 receptor is Flk1.

4. The isolated nucleic acid of claim 1 wherein the second VEGF receptor is Flt4.

25

5. The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.
6. The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.
7. The isolated nucleic acid molecule of claim 1, wherein the multimerizing component comprises an immunoglobulin domain.
8. The isolated nucleic acid molecule of claim 1, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.
9. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of:
- (a) the nucleotide sequence set forth in Figure 13A-13D;
  - (b) the nucleotide sequence set forth in Figure 14A-14C;

- (c) the nucleotide sequence set forth in Figure 15A-15C;  
(d) the nucleotide sequence set forth in Figure 16A-16D;  
(e) the nucleotide sequence set forth in Figure 21A-21C  
(f) the nucleotide sequence set forth in Figure 22A-22C;  
5 (g) the nucleotide sequence set forth in Figure 24A-24C; and  
(h) a nucleotide sequence which, as a result of the degeneracy of  
the genetic code, differs from the nucleotide sequence of (a), (b), (c ),  
(d), (e), (f), or (g) and which encodes a fusion polypeptide molecule  
having the biological activity of the modified Flt1 receptor fusion  
10 polypeptide.

10. A fusion polypeptide encoded by the isolated nucleic acid molecule  
of claim 1, 2, 3, 4 or 9.

- 15 11. A composition capable of binding a VEGF molecule to form a  
nonfunctional complex comprising a multimer of the fusion polypeptide  
of claim 10.

12. The composition of claim 11, wherein the multimer is a dimer.

20

13. The composition of claim 12 and a carrier.

14. A vector which comprises the nucleic acid molecule of claim 1, 2,  
3, 4 or 9.

25

15. An expression vector comprising a nucleic acid molecule of claim 1, 2, 3, 4 or 9 wherein the nucleic acid molecule is operatively linked to an expression control sequence.

5 16. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 15, in a suitable host cell.

17. The host-vector system of claim 16, wherein the suitable host cell  
10 is a bacterial cell, yeast cell, insect cell, or mammalian cell.

18. The host-vector system of claim 16, wherein the suitable host cell is E. coli.

15 19. The host-vector system of claim 16, wherein the suitable host cell is a COS cell.

20. The host-vector system of claim 14, wherein the suitable host cell is a CHO cell.

20

21. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claim 16, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

25

22. A fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D or Figure 24A-24C, which has been modified by acetylation or pegylation.

5 23. The fusion polypeptide of claim 22 wherein the modification is acetylation.

24. The fusion polypeptide of claim 22 wherein the modification is pegylation.

10

25. The fusion polypeptide of claim 23 wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent.

15 26. The fusion polypeptide of claim 23 wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess.

20 27. The fusion polypeptide of claim 24 wherein the pegylation is 10K or 20K PEG.

28. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 10.

25



29. The method of claim 28, wherein the mammal is a human.

30. The method of claim 29, wherein the fusion polypeptide is acetylated.

5

31. The method of claim 29, wherein the fusion polypeptide is pegylated.

32. The fusion polypeptide of claims 10 which specifically binds the  
10 VEGF receptor ligand VEGF.

33. A method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide of claim  
10.

15

34. A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide of claim 10.

20 35. The method of claim 34, wherein the mammal is a human.

36. The method of claim 34, used to attenuate or prevent tumor growth in a human.

25

37. The method of claim 34, used to attenuate or prevent edema in a human.

38. The method of claim 34, used to attenuate or prevent ascites  
5 formation in a human.

39. The method of claim 37, wherein the edema is brain edema.

40. The method of claim 38, wherein the ascites is ovarian cancer  
10 associated ascites.

41. A fusion polypeptide capable of binding a VEGF polypeptide comprising:

(a) a VEGF receptor component operatively linked to

15 (b) a multimerizing component,

wherein the VEGF receptor component is the only VEGF receptor component in the fusion polypeptide and consists essentially of the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and the amino acid sequence of Ig domain 3 of the  
20 extracellular domain of a second VEGF receptor.

42. The fusion polypeptide of claim 41 wherein the first VEGF receptor is Flt1.

43. The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flk1.

44. The fusion polypeptide of claim 41 wherein the second VEGF  
5 receptor is Flt4.

45. The fusion polypeptide claim 41, wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular  
10 domain of the second VEGF receptor.

46. The fusion polypeptide of claim 41, wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of  
15 the extracellular domain of the second VEGF receptor.

47. The fusion polypeptide of claim 41, wherein the multimerizing component comprises an immunoglobulin domain.

20 48. The fusion polypeptide of claim 41, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

49. An fusion polypeptide comprising an amino acid sequence of a  
25 modified Flt1 receptor, wherein the amino acid sequence selected from

the group consisting of:

- (a) the amino acid sequence set forth in Figure 13A-13D;
- (b) the amino acid sequence set forth in Figure 14A-14C;
- (c) the amino acid sequence set forth in Figure 15A-15C;
- 5 (d) the amino acid sequence set forth in Figure 16A-16D;
- (e) the amino acid sequence set forth in Figure 21A-21C
- (f) the amino acid sequence set forth in Figure 22A-22C; and
- (g) the amino acid sequence set forth in Figure 24A-24C.

10 50. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 41, 42, 43, 44 or 49.

15 51. A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide of claim 41, 42, 43, 44 or 49.

1/55

Fig.1.

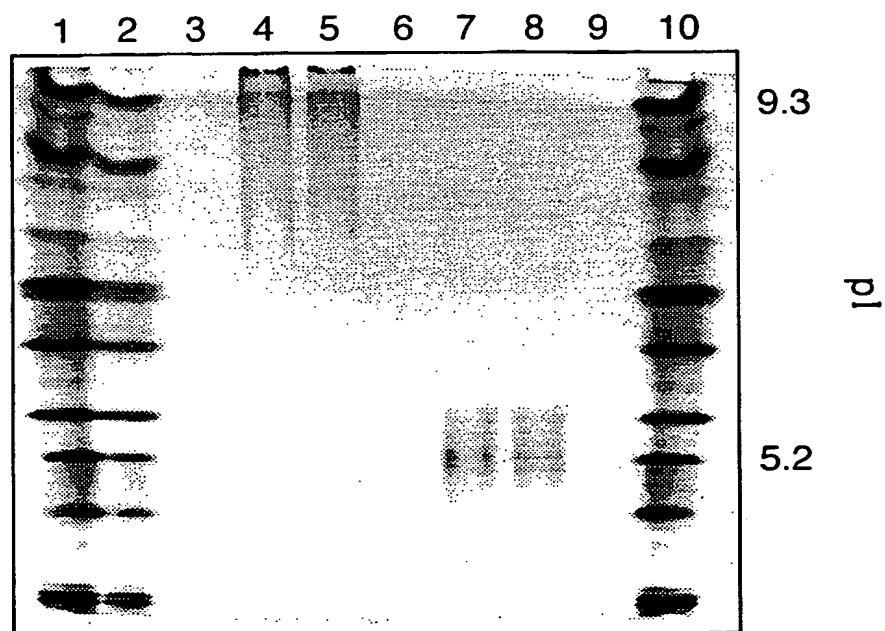


Fig.2.

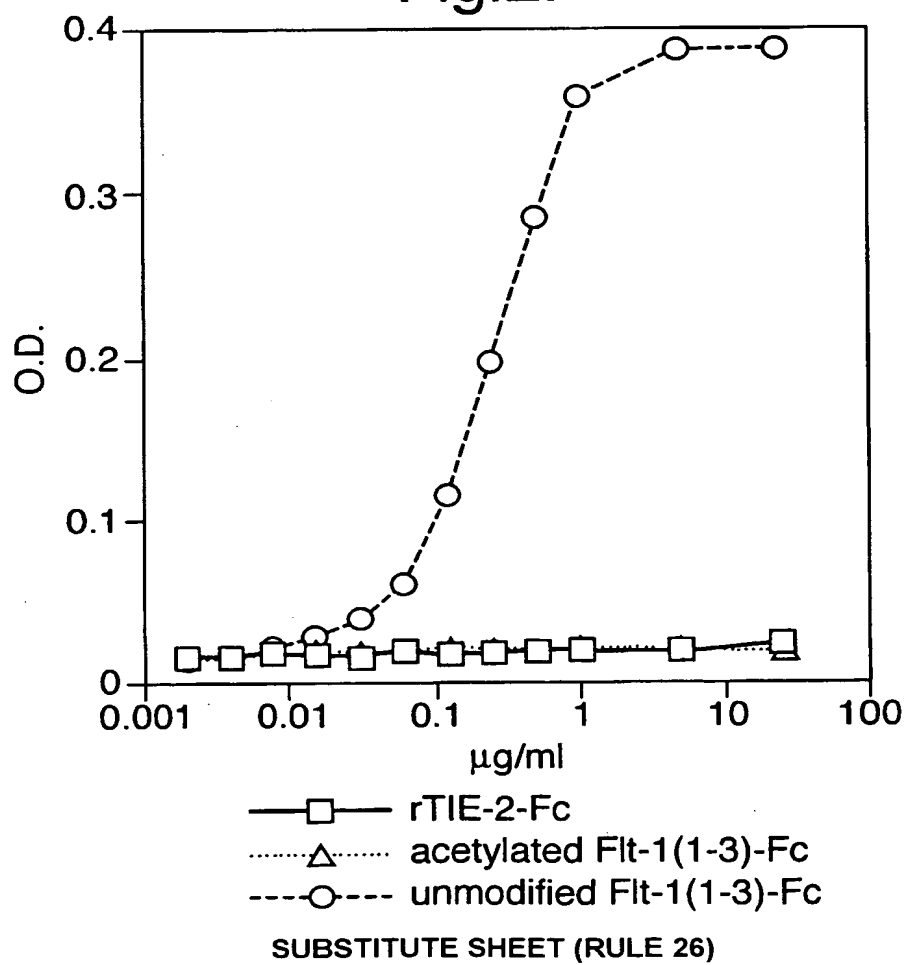
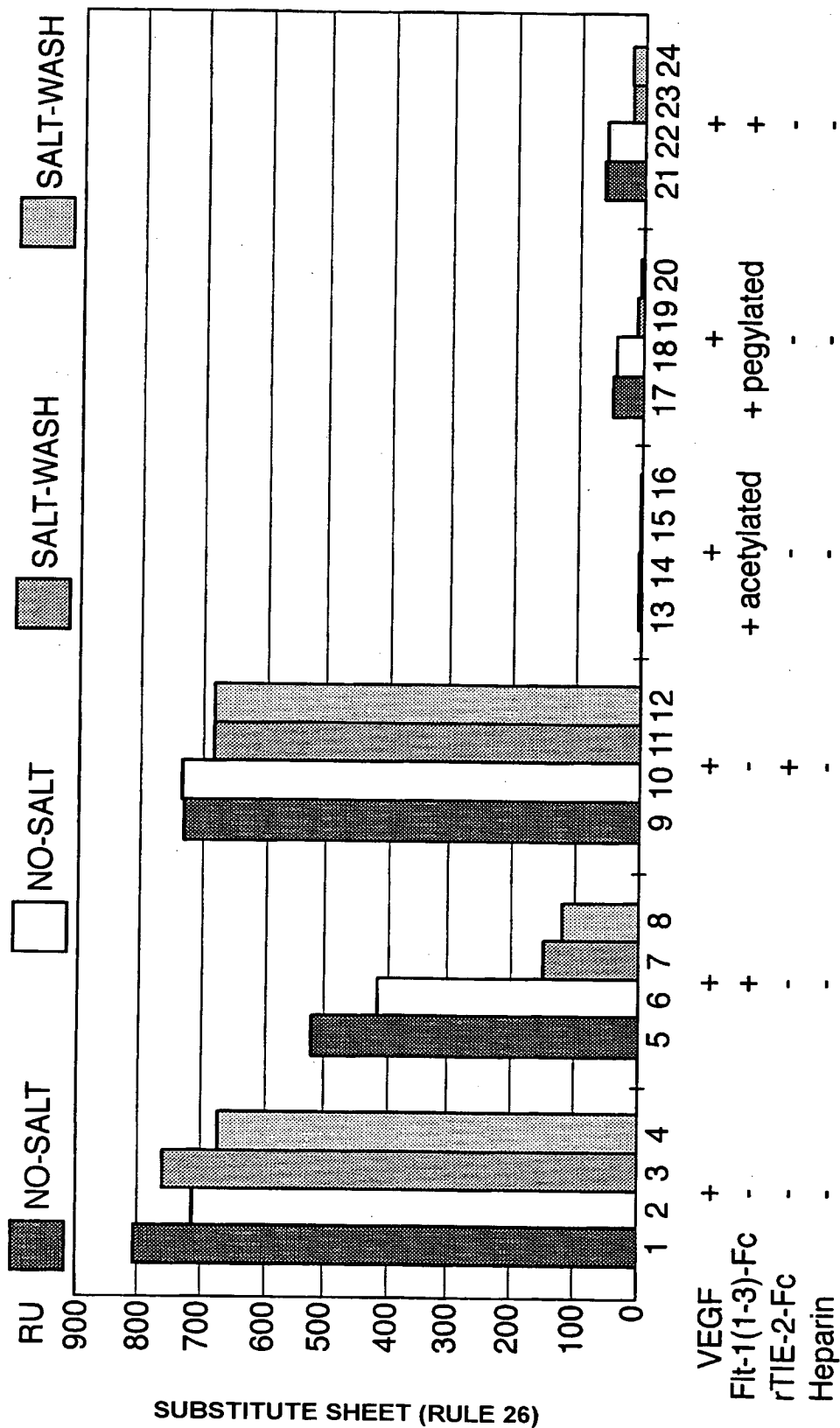


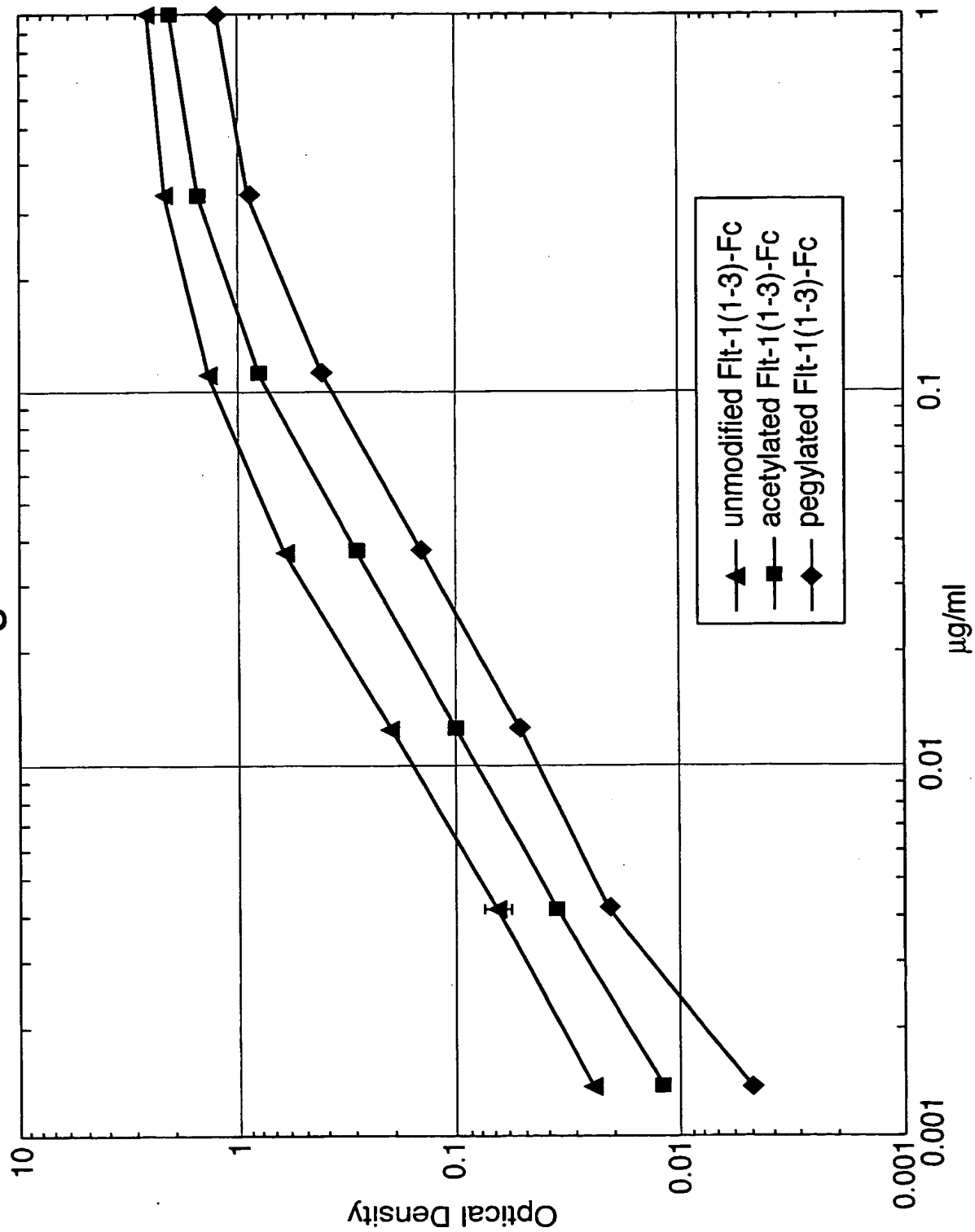
Fig.3.



SUBSTITUTE SHEET (RULE 26)

3/55

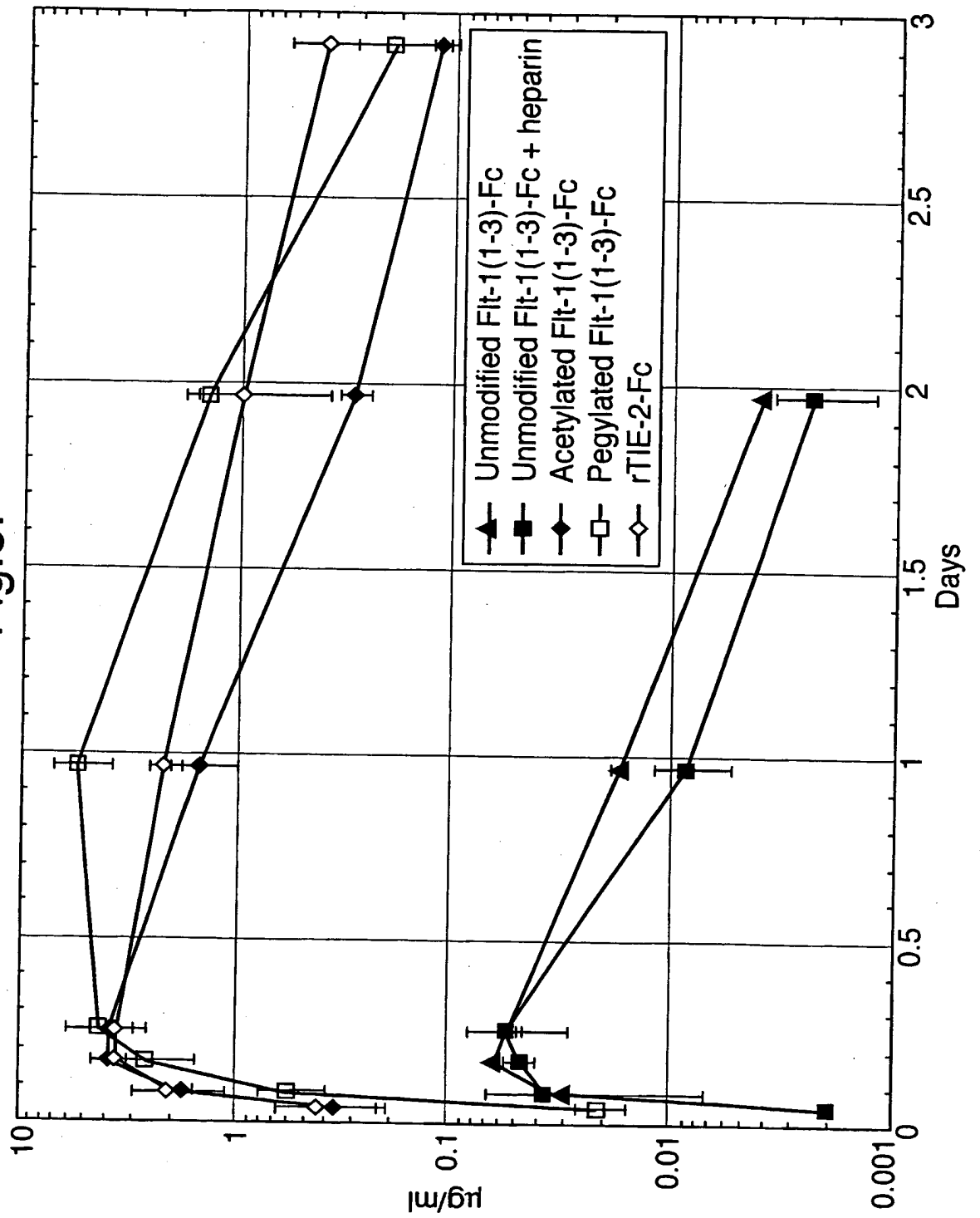
Fig.4.



SUBSTITUTE SHEET (RULE 26)

4/55

Fig.5.



SUBSTITUTE SHEET (RULE 26)



5/55

Fig.6A.

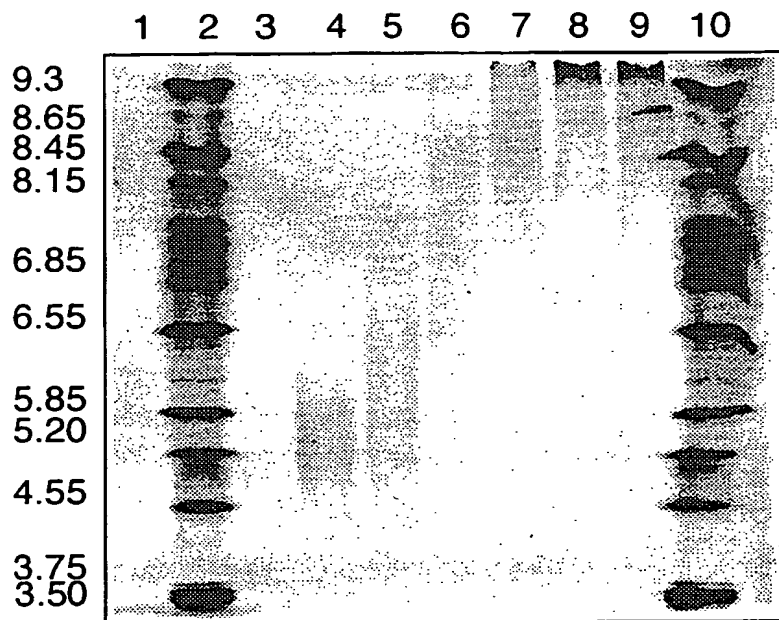
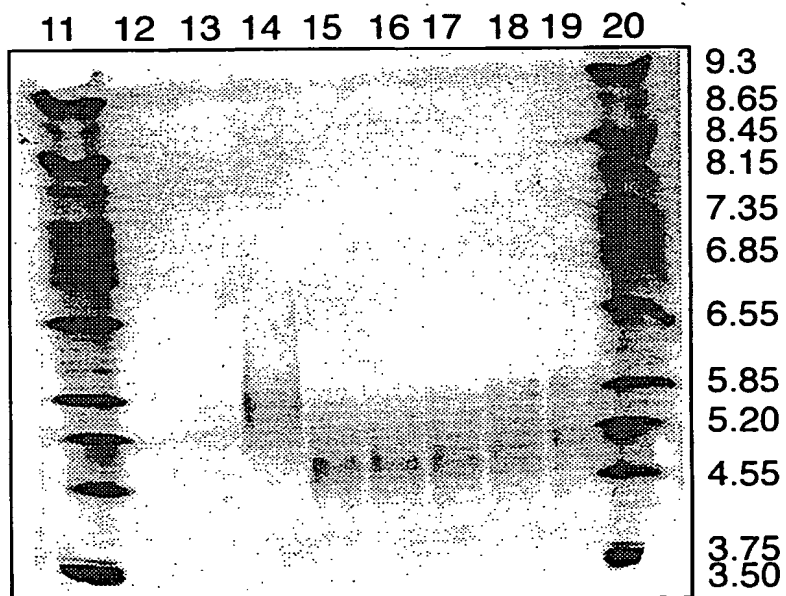


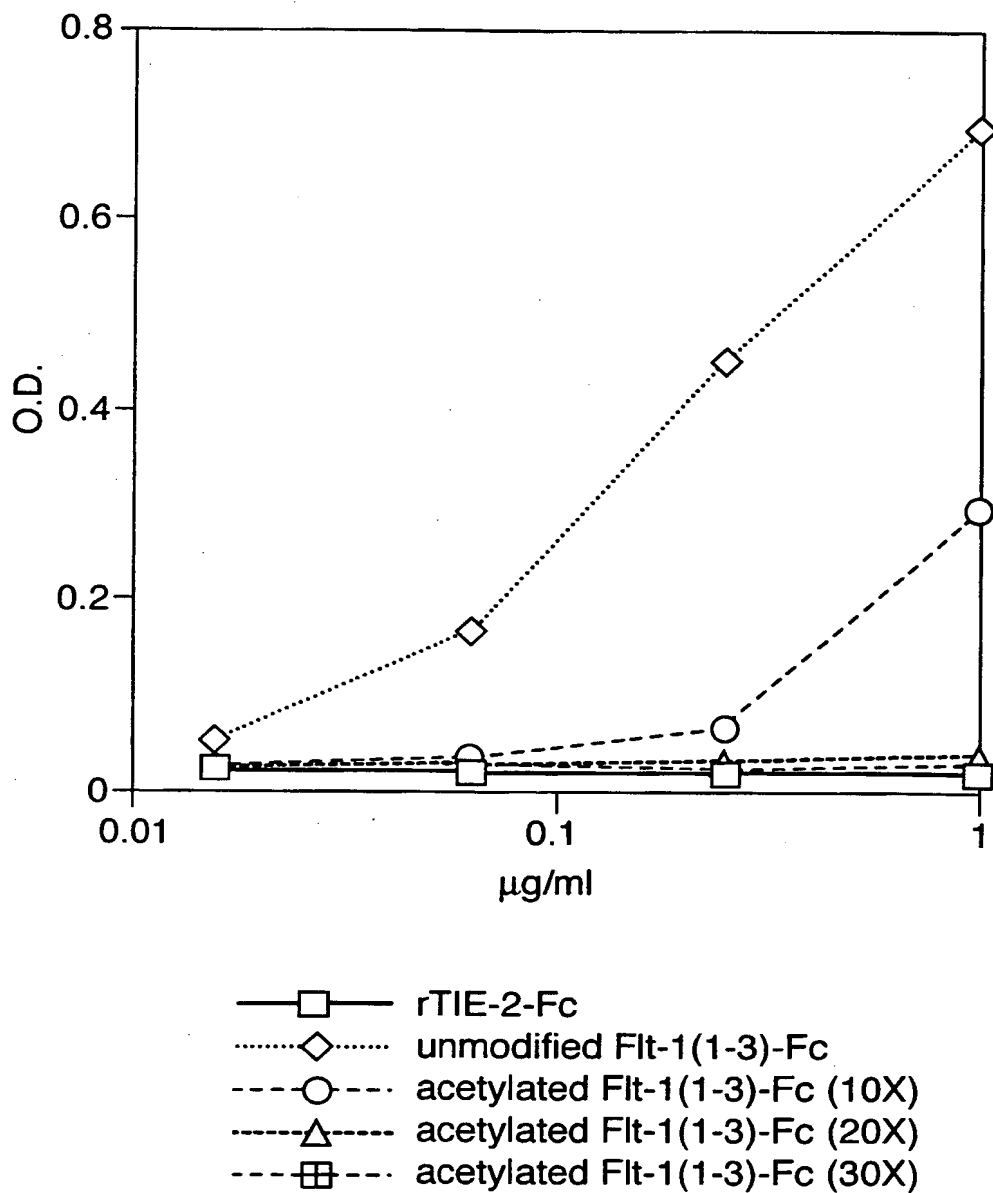
Fig.6B.



SUBSTITUTE SHEET (RULE 26)

6/55

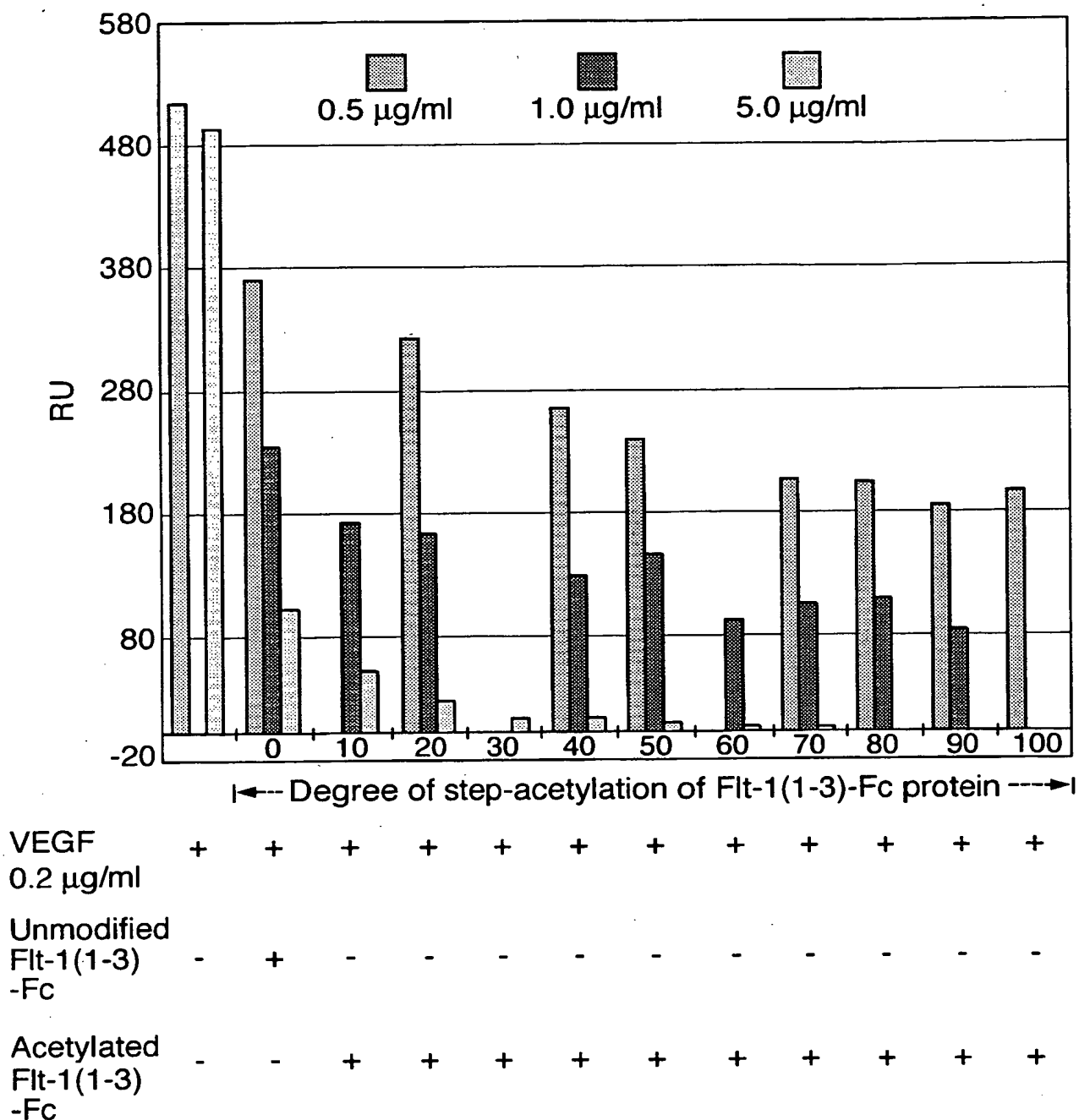
Fig.7.



SUBSTITUTE SHEET (RULE 26)

7/55

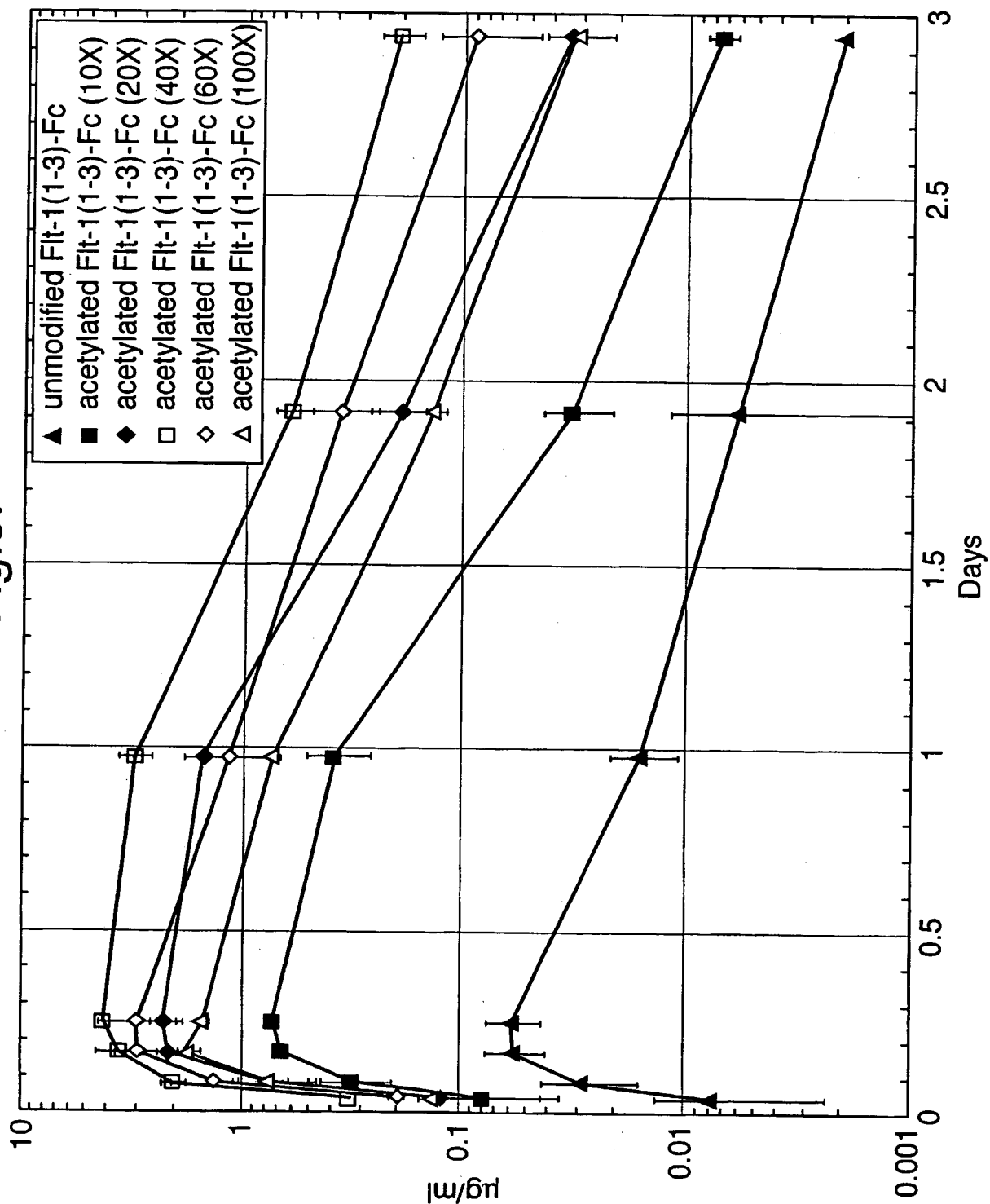
Fig.8.



SUBSTITUTE SHEET (RULE 26)

8/55

Fig.9.



SUBSTITUTE SHEET (RULE 26)

9/55

## Fig.10A.

```

      10      20      30      40      50      60
      *      *      *      *      *      *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

      70      80      90      100      110      120
      *      *      *      *      *      *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

      130      140      150      160      170      180
      *      *      *      *      *      *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

      190      200      210      220      230      240
      *      *      *      *      *      *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CCG
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

      250      260      270      280      290      300
      *      *      *      *      *      *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

      310      320      330      340      350      360
      *      *      *      *      *      *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

      370      380      390      400      410      420
      *      *      *      *      *      *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

      430      440      450      460      470      480
      *      *      *      *      *      *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

      490      500      510      520      530      540
      *      *      *      *      *      *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>

```

SUBSTITUTE SHEET (RULE 26)

10/55

## Fig.10B.

```

      550      560      570      580      590      600
      *      *      *      *      *      *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

      610      620      630      640      650      660
      *      *      *      *      *      *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

      670      680      690      700      710      720
      *      *      *      *      *      *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>

      730      740      750      760      770      780
      *      *      *      *      *      *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>

      790      800      810      820      830      840
      *      *      *      *      *      *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg>

      850      860      870      880      890      900
      *      *      *      *      *      *
CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA
GCT TAA CTG GTT TCG TTA AGG GTA CCG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT
Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>

      910      920      930      940      950      960
      *      *      *      *      *      *
ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA
TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT
Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys>

      970      980      990      1000      1010      1020
      *      *      *      *      *      *
TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT
AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>

      1030      1040      1050      1060      1070      1080
      *      *      *      *      *      *
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>

```

SUBSTITUTE SHEET (RULE 26)

11/55

## Fig.10C.

1090			1100			1110			1120			1130			1140		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TTC	CTC	TTC	CCC	CCA	AAA	CCC	AAG	GAC	ACC	CTC	ATG	ATC	TCC	CGG	ACC	CCT	
AAG	GAG	AAG	GGG	GGT	TTT	GGG	TTC	CTG	TGG	GAG	TAC	TAG	AGG	GCC	TGG	GGA	
Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	
1150			1160			1170			1180			1190			1200		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	
ACG	CAC	CAC	CAC	CTG	CAC	TCG	GTG	CTT	CTG	GGA	CTC	CAG	TTC	AAG	TTG	ACC	
Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	
1210			1220			1230			1240			1250			1260		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	
CCG	CAC	CTC	CAC	GTA	TTA	CGG	TTC	TGT	TTC	GGC	GCC	CTC	CTC	GTC	ATG	TTG	
Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	
1270			1280			1290			1300			1310			1320		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
CGT	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC	TGG	CTG	AAT	GGC	AAG	
GCA	CAC	CAG	TCG	CAG	GAG	TGG	CAG	GAC	GTG	GTC	CTG	ACC	GAC	TTA	CCG	TTC	
Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	
1330			1340			1350			1360			1370			1380		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TGC	AAG	GTC	TCC	AAC	AAA	GCC	CTC	CCA	GCC	CCC	ATC	GAG	AAA	ACC	ATC	TCC	
ACG	TTC	CAG	AGG	TTG	TTT	CGG	GAG	GGT	CGG	GGG	TAG	CTC	TTT	TGG	TAG	AGG	
Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	
1390			1400			1410			1420			1430			1440		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GGG	CAG	CCC	CGA	GAA	CCA	CAG	GTG	TAC	ACC	CTG	CCC	CCA	TCC	CGG	GAT	GAG	
CCC	GTC	GGG	GCT	CTT	GGT	GTC	CAC	ATG	TGG	GAC	GGG	GGT	AGG	GCC	CTA	CTC	
Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	
1450			1460			1470			1480			1490			1500		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	GGC	TTC	TAT	CCC	AGC	GAC	ATC	
TTG	GTC	CAG	TCG	GAC	TGG	ACG	GAC	CAG	TTT	CCG	AAG	ATA	GGG	TCG	CTG	TAG	
Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	
1510			1520			1530			1540			1550			1560		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TGG	GAG	AGC	AAT	GGG	CAG	CCG	GAG	AAC	AAC	TAC	AAG	ACC	ACG	CCT	CCC	GTG	
ACC	CTC	TCG	TTA	CCC	GTC	GGC	CTC	TTG	TTG	ATG	TTC	TGG	TGC	GGA	GGG	CAC	
Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	
1570			1580			1590			1600			1610			1620		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GAC	GGC	TCC	TTC	TTC	CTC	TAC	AGC	AAG	CTC	ACC	GTG	GAC	AAG	AGC	AGG	TGG	
CTG	CCG	AGG	AAG	AAG	GAG	ATG	TCG	TTC	GAG	TGG	CAC	CTG	TTC	TCG	TCC	ACC	
Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	

SUBSTITUTE SHEET (RULE 26)

12/55

## Fig.10D.

```

      1630      1640      1650      1660      1670      1680
      *        *        *        *        *        *
AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC
TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser>

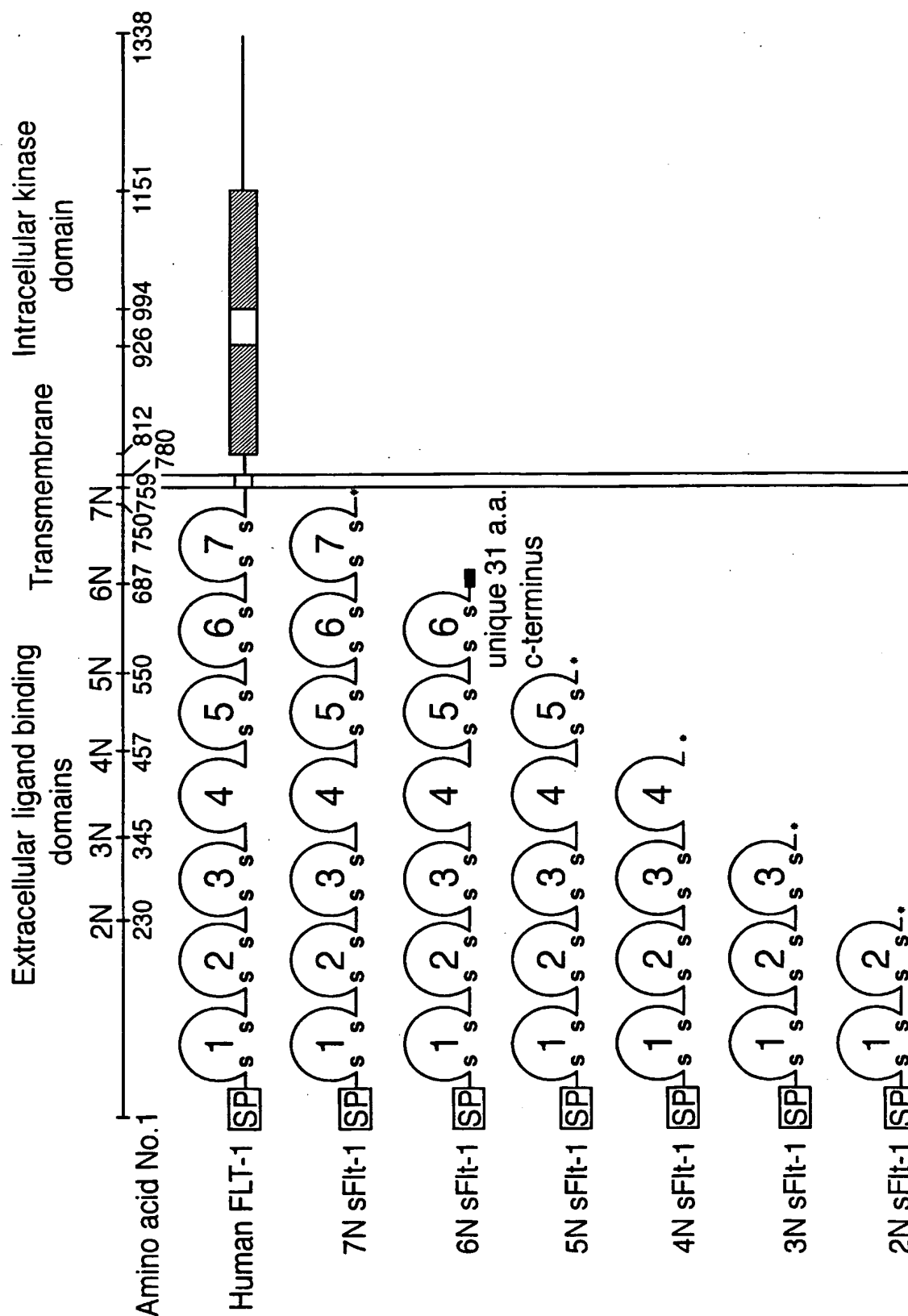
      1690      1700
      *        *        *        *
CTC TCC CTG TCT CCG GGT AAA TGA
GAG AGG GAC AGA GGC CCA TTT ACT
Leu Ser Leu Ser Pro Gly Lys ***>

```

SUBSTITUTE SHEET (RULE 26)



Fig.11.



SUBSTITUTE SHEET (RULE 26)

Fig. 12A.

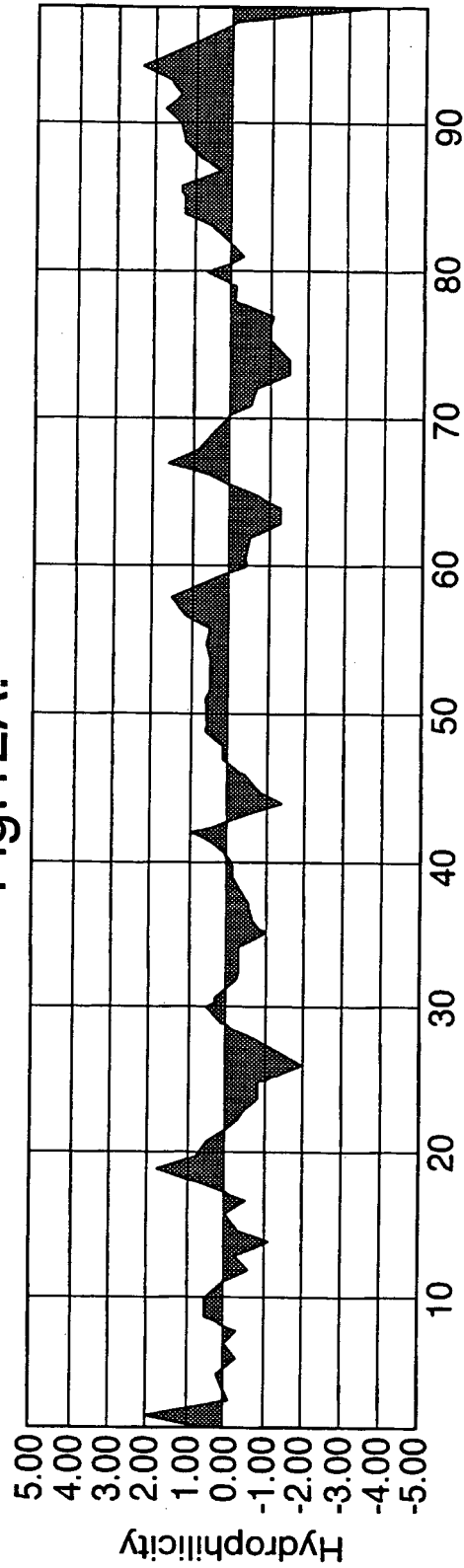
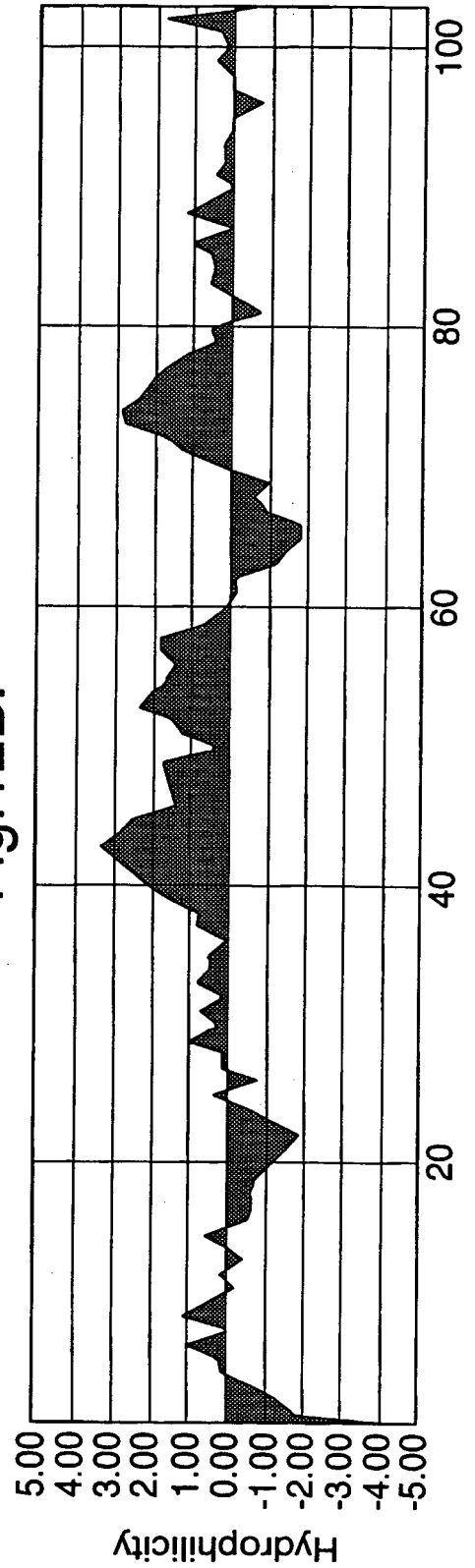


Fig. 12B.



SUBSTITUTE SHEET (RULE 26)

15/55

## Fig.13A.

			10				20				30				40				50				60
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ATG	GTC	AGC	TAC	TGG	GAC	ACC	GGG	GTC	CTG	CTG	TGC	GCG	CTG	CTC	AGC	TGT	CTG	CTT	CTC				
TAC	CAG	TCG	ATG	ACC	CTG	TGG	CCC	CAG	GAC	GAC	ACG	CGC	GAC	GAG	TCG	ACA	GAC	GAA	GAG				
Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu				
			70				80				90				100				110				120
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ACA	GGA	TCT	AGT	TCA	GGT	TCA	AAA	TTA	AAA	GAT	CCT	GAA	CTG	AGT	TTA	AAA	GGC	ACC	CAG				
TGT	CCT	AGA	TCA	AGT	CCA	AGT	TTT	AAT	TTT	CTA	GGA	CTT	GAC	TCA	AAT	TTT	CCG	TGG	GTC				
Thr	Gly	Ser	Ser	Ser	Gly	Ser	Lys	Leu	Lys	Asp	Pro	Glu	Leu	Ser	Leu	Lys	Gly	Thr	Gln				
			130				140				150				160				170				180
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CAC	ATC	ATG	CAA	GCA	GGC	CAG	ACA	CTG	CAT	CTC	CAA	TGC	AGG	GGG	GAA	GCA	GCC	CAT	AAA				
GTG	TAG	TAC	GTT	CGT	CCG	GTC	TGT	GAC	GTA	GAG	GTT	ACG	TCC	CCC	CTT	CGT	CGG	GTA	TTT				
His	Ile	Met	Gln	Ala	Gly	Gln	Thr	Leu	His	Leu	Gln	Cys	Arg	Gly	Glu	Ala	Ala	His	Lys				
			190				200				210				220				230				240
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TGG	TCT	TTG	CCT	GAA	ATG	GTG	AGT	AAG	GAA	AGC	GAA	AGG	CTG	AGC	ATA	ACT	AAA	TCT	GCC				
ACC	AGA	AAC	GGA	CTT	TAC	CAC	TCA	TTC	CTT	TCG	CTT	TCC	GAC	TCG	TAT	TGA	TTT	AGA	CGG				
Trp	Ser	Leu	Pro	Glu	Met	Val	Ser	Lys	Glu	Ser	Glu	Arg	Leu	Ser	Ile	Thr	Lys	Ser	Ala				
			250				260				270				280				290				300
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TGT	GGA	AGA	AAT	GGC	AAA	CAA	TTC	TGC	AGT	ACT	TTA	ACC	TTG	AAC	ACA	GCT	CAA	GCA	AAC				
ACA	CCT	TCT	TTA	CCG	TTT	GTT	AAG	ACG	TCA	TGA	AAT	TGG	AAC	TTG	TGT	CGA	GTT	CGT	TTG				
Cys	Gly	Arg	Asn	Gly	Lys	Gln	Phe	Cys	Ser	Thr	Leu	Thr	Leu	Asn	Thr	Ala	Gln	Ala	Asn				
			310				320				330				340				350				360
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CAC	ACT	GGC	TTC	TAC	AGC	TGC	AAA	TAT	CTA	GCT	GTA	CCT	ACT	TCA	AAG	AAG	AAG	GAA	ACA				
GTG	TGA	CCG	AAG	ATG	TCG	ACG	TTT	ATA	GAT	CGA	CAT	GGA	TGA	AGT	TTC	TTC	TTC	CTT	TGT				
His	Thr	Gly	Phe	Tyr	Ser	Cys	Lys	Tyr	Leu	Ala	Val	Pro	Thr	Ser	Lys	Lys	Lys	Glu	Thr				
			370				380				390				400				410				420
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAA	TCT	GCA	ATC	TAT	ATA	TTT	ATT	AGT	GAT	ACA	GGT	AGA	CCT	TTC	GTA	GAG	ATG	TAC	AGT				
CTT	AGA	CGT	TAG	ATA	TAT	AAA	TAA	TCA	CTA	TGT	CCA	TCT	GGA	AAG	CAT	CTC	TAC	ATG	TCA				
Glu	Ser	Ala	Ile	Tyr	Ile	Phe	Ile	Ser	Asp	Thr	Gly	Arg	Pro	Phe	Val	Glu	Met	Tyr	Ser				
			430				440				450				460				470				480
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAA	ATC	CCC	GAA	ATT	ATA	CAC	ATG	ACT	GAA	GGA	AGG	GAG	CTC	GTC	ATT	CCC	TGC	CGG	GTT				
CTT	TAG	GGG	CTT	TAA	TAT	GTG	TAC	TGA	CTT	CCT	TCC	CTC	GAG	CAG	TAA	GGG	ACG	GCC	CAA				
Glu	Ile	Pro	Glu	Ile	Ile	His	Met	Thr	Glu	Gly	Arg	Glu	Leu	Val	Ile	Pro	Cys	Arg	Val				
			490				500				510				520				530				540
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ACG	TCA	CCT	AAC	ATC	ACT	GTT	ACT	TTA	AAA	AAG	TTT	CCA	CTT	GAC	ACT	TTG	ATC	CCT	GAT				
TGC	AGT	GGA	TTG	TAG	TGA	CAA	TGA	AAT	TTT	TTC	AAA	GGT	GAA	CTG	TGA	AAC	TAG	GGA	CTA				
Thr	Ser	Pro	Asn	Ile	Thr	Val	Thr	Leu	Lys	Lys	Phe	Pro	Leu	Asp	Thr	Leu	Ile	Pro	Asp				

SUBSTITUTE SHEET (RULE 26)

16/55

## Fig.13B.

```

      550      560      570      580      590      600
      *      *      *      *      *      *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

      610      620      630      640      650      660
      *      *      *      *      *      *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

      670      680      690      700      710      720
      *      *      *      *      *      *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>

      730      740      750      760      770      780
      *      *      *      *      *      *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>

      790      800      810      820      830      840
      *      *      *      *      *      *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AAC
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG GTA CGG TTG
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn>

      850      860      870      880      890      900
      *      *      *      *      *      *
ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT
TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA
Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr>

      910      920      930      940      950      960
      *      *      *      *      *      *
TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT
ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA
Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp>

      970      980      990      1000      1010      1020
      *      *      *      *      *      *
AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA
TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro>

      1030      1040      1050      1060      1070      1080
      *      *      *      *      *      *
GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC
CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr>

```

SUBSTITUTE SHEET (RULE 26)

17/55

## Fig.13C.

1090	1100	1110	1120	1130	1140
* * *	* *	* *	* *	* *	* *
CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC					
GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG					
Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp>					
1150	1160	1170	1180	1190	1200
* * *	* *	* *	* *	* *	* *
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG					
GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC					
Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys>					
1210	1220	1230	1240	1250	1260
* * *	* *	* *	* *	* *	* *
CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC					
GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG					
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His>					
1270	1280	1290	1300	1310	1320
* * *	* *	* *	* *	* *	* *
CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC					
GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG					
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala>					
1330	1340	1350	1360	1370	1380
* * *	* *	* *	* *	* *	* *
CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC					
GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG					
Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr>					
1390	1400	1410	1420	1430	1440
* * *	* *	* *	* *	* *	* *
CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA					
GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT					
Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys>					
1450	1460	1470	1480	1490	1500
* * *	* *	* *	* *	* *	* *
GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC					
CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG					
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn>					
1510	1520	1530	1540	1550	1560
* * *	* *	* *	* *	* *	* *
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC					
ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG					
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu>					
1570	1580	1590	1600	1610	1620
* * *	* *	* *	* *	* *	* *
ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG					
TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC					
Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu>					

SUBSTITUTE SHEET (RULE 26)

18/55

## Fig.13D.

		1630			1640				1650			1660			1670	7
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CTG	TCT	CCG	GGT	AAA
CGA	GAC	GTG	TTG	GTG	ATG	TGC	GTC	TTC	TCG	GAG	AGG	GAC	AGA	GGC	CCA	TTT
Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly	Lys
																***>

SUBSTITUTE SHEET (RULE 26)

19/55

## Fig.14A.

			10				20				30				40				50				60
	*		*		*		*		*		*		*		*		*		*		*		*
ATG	GTC	AGC	TAC	TGG	GAC	ACC	GGG	GTC	CTG	CTG	TGC	GCG	CTG	CTC	AGC	TGT	CTG	CTT	CTC				
TAC	CAG	TCG	ATG	ACC	CTG	TGG	CCC	CAG	GAC	GAC	ACG	CGC	GAC	GAG	TCG	ACA	GAC	GAA	GAG				
Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu	>			
			70				80				90				100				110				120
	*		*		*		*		*		*		*		*		*		*		*		*
ACA	GGA	TCT	AGT	TCC	GGA	GGT	AGA	CCT	TTC	GTA	GAG	ATG	TAC	AGT	GAA	ATC	CCC	GAA	ATT				
TGT	CCT	AGA	TCA	AGG	CCT	CCA	TCT	GGA	AAG	CAT	CTC	TAC	ATG	TCA	CTT	TAG	GGG	CTT	TAA				
Thr	Gly	Ser	Ser	Ser	Gly	Gly	Arg	Pro	Phe	Val	Glu	Met	Tyr	Ser	Glu	Ile	Pro	Glu	Ile	>			
			130				140				150				160				170				180
	*		*		*		*		*		*		*		*		*		*		*		*
ATA	CAC	ATG	ACT	GAA	GGA	AGG	GAG	CTC	GTC	ATT	CCC	TGC	CGG	GTT	ACG	TCA	CCT	AAC	ATC				
TAT	GTG	TAC	TGA	CTT	CCT	TCC	CTC	GAG	CAG	TAA	GGG	ACG	GCC	CAA	TGC	AGT	GGA	TTG	TAG				
Ile	His	Met	Thr	Glu	Gly	Arg	Glu	Leu	Val	Ile	Pro	Cys	Arg	Val	Thr	Ser	Pro	Asn	Ile	>			
			190				200				210				220				230				240
	*		*		*		*		*		*		*		*		*		*		*		*
ACT	GTT	ACT	TTA	AAA	AAG	TTT	CCA	CTT	GAC	ACT	TTG	ATC	CCT	GAT	GGA	AAA	CGC	ATA	ATC				
TGA	CAA	TGA	AAT	TTT	TTC	AAA	GGT	GAA	CTG	TGA	AAC	TAG	GGA	CTA	CCT	TTT	GCG	TAT	TAG				
Thr	Val	Thr	Leu	Lys	Lys	Phe	Pro	Leu	Asp	Thr	Leu	Ile	Pro	Asp	Gly	Lys	Arg	Ile	Ile	>			
			250				260				270				280				290				300
	*		*		*		*		*		*		*		*		*		*		*		*
TGG	GAC	AGT	AGA	AAG	GGC	TTC	ATC	ATA	TCA	AAT	GCA	ACG	TAC	AAA	GAA	ATA	GGG	CTT	CTG				
ACC	CTG	TCA	TCT	TTC	CCG	AAG	TAG	TAT	AGT	TTA	CGT	TGC	ATG	TTT	CTT	TAT	CCC	GAA	GAC				
Trp	Asp	Ser	Arg	Lys	Gly	Phe	Ile	Ile	Ser	Asn	Ala	Thr	Tyr	Lys	Glu	Ile	Gly	Leu	Leu	>			
			310				320				330				340				350				360
	*		*		*		*		*		*		*		*		*		*		*		*
ACC	TGT	GAA	GCA	ACA	GTC	AAT	GGG	CAT	TTG	TAT	AAG	ACA	AAC	TAT	CTC	ACA	CAT	CGA	CAA				
TGG	ACA	CTT	CGT	TGT	CAG	TTA	CCC	GTA	AAC	ATA	TTC	TGT	TTG	ATA	GAG	TGT	GTA	GCT	GTT				
Thr	Cys	Glu	Ala	Thr	Val	Asn	Gly	His	Leu	Tyr	Lys	Thr	Asn	Tyr	Leu	Thr	His	Arg	Gln	>			
			370				380				390				400				410				420
	*		*		*		*		*		*		*		*		*		*		*		*
ACC	AAT	ACA	ATC	ATA	GAT	GTC	CAA	ATA	AGC	ACA	CCA	CGC	CCA	GTC	AAA	TTA	CTT	AGA	GGC				
TGG	TTA	TGT	TAG	TAT	CTA	CAG	GTT	TAT	TCG	TGT	GGT	GCG	GGT	CAG	TTT	AAT	GAA	TCT	CCG				
Thr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Ser	Thr	Pro	Arg	Pro	Val	Lys	Leu	Leu	Arg	Gly	>			
			430				440				450				460				470				480
	*		*		*		*		*		*		*		*		*		*		*		*
CAT	ACT	CTT	GTC	CTC	AAT	TGT	ACT	GCT	ACC	ACT	CCC	TTG	AAC	ACG	AGA	GTT	CAA	ATG	ACC				
GTA	TGA	GAA	CAG	GAG	TTA	ACA	TGA	CGA	TGG	TGA	GGG	AAC	TTG	TGC	TCT	CAA	GTT	TAC	TGG				
His	Thr	Leu	Val	Leu	Asn	Cys	Thr	Ala	Thr	Thr	Pro	Leu	Asn	Thr	Arg	Val	Gln	Met	Thr	>			
			490				500				510				520				530				540
	*		*		*		*		*		*		*		*		*		*		*		*
TGG	AGT	TAC	CCT	GAT	GAA	ATT	GAC	CAA	AGC	AAT	TCC	CAT	GCC	AAC	ATA	TTC	TAC	AGT	GTT				
ACC	TCA	ATG	GGA	CTA	CTT	TAA	CTG	GTT	TCG	TTA	AGG	GTA	CGG	TTG	TAT	AAG	ATG	TCA	CAA				
Trp	Ser	Tyr	Pro	Asp	Glu	Ile	Asp	Gln	Ser	Asn	Ser	His	Ala	Asn	Ile	Phe	Tyr	Ser	Val	>			

SUBSTITUTE SHEET (RULE 26)

20/55

## Fig.14B.

```

      550      560      570      580      590      600
      *      *      *      *      *      *
CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT
GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA
Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser>

      610      620      630      640      650      660
      *      *      *      *      *      *
GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC
CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG
Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly>

      670      680      690      700      710      720
      *      *      *      *      *      *
GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG
CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC
Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu>

      730      740      750      760      770      780
      *      *      *      *      *      *
GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG
CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg>

      790      800      810      820      830      840
      *      *      *      *      *      *
ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC
TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe>

      850      860      870      880      890      900
      *      *      *      *      *      *
AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG
TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln>

      910      920      930      940      950      960
      *      *      *      *      *      *
TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT
ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA
Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn>

      970      980      990      1000      1010      1020
      *      *      *      *      *      *
GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>

      1030      1040      1050      1060      1070      1080
      *      *      *      *      *      *
ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG
TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg>

```

SUBSTITUTE SHEET (RULE 26)



21/55

## Fig.14C.

1090	1100	1110	1120	1130	1140
* * *	* *	* *	* *	* *	* *
GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC					
CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG					
Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser>					
1150	1160	1170	1180	1190	1200
* * *	* *	* *	* *	* *	* *
GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT					
CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA					
Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro>					
1210	1220	1230	1240	1250	1260
* * *	* *	* *	* *	* *	* *
CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC					
GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG					
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser>					
1270	1280	1290	1300	1310	1320
* * *	* *	* *	* *	* *	* *
AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC					
TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG					
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His>					
1330	1340	1350			
* * *	* *	* *			
TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA					
ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT					
Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>					

SUBSTITUTE SHEET (RULE 26)

22/55

Fig.15A.

	10		20		30		40		50		60								
*	*	*	*	*	*	*	*	*	*	*	*								
ATG	GTC	AGC	TAC	TGG	GAC	ACC	GGG	GTC	CTG	CTG	TGC	GCG	CTG	CTC	AGC	TGT	CTG	CTT	CTC
TAC	CAG	TCG	ATG	ACC	CTG	TGG	CCC	CAG	GAC	GAC	ACG	CGC	GAC	GAG	TCG	ACA	GAC	GAA	GAG
Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu>
	70		80		90		100		110		120								
*	*	*	*	*	*	*	*	*	*	*	*								
ACA	GGA	TCT	AGT	TCC	GGA	GGT	AGA	CCT	TTC	GTA	GAG	ATG	TAC	AGT	GAA	ATC	CCC	GAA	ATT
TGT	CCT	AGA	TCA	AGG	CCT	CCA	TCT	GGA	AAG	CAT	CTC	TAC	ATG	TCA	CTT	TAG	GGG	CTT	TAA
Thr	Gly	Ser	Ser	Ser	Gly	Gly	Arg	Pro	Phe	Val	Glu	Met	Tyr	Ser	Glu	Ile	Pro	Glu	Ile>
	130		140		150		160		170		180								
*	*	*	*	*	*	*	*	*	*	*	*								
ATA	CAC	ATG	ACT	GAA	GGA	AGG	GAG	CTC	GTC	ATT	CCC	TGC	CGG	GTT	ACG	TCA	CCT	AAC	ATC
TAT	GTG	TAC	TGA	CTT	CCT	TCC	CTC	GAG	CAG	TAA	GGG	ACG	GCC	CAA	TGC	AGT	GGA	TTG	TAG
Ile	His	Met	Thr	Glu	Gly	Arg	Glu	Leu	Val	Ile	Pro	Cys	Arg	Val	Thr	Ser	Pro	Asn	Ile>
	190		200		210		220		230		240								
*	*	*	*	*	*	*	*	*	*	*	*								
ACT	GTT	ACT	TTA	AAA	AAG	TTT	CCA	CTT	GAC	ACT	TTG	ATC	CCT	GAT	GGA	AAA	CGC	ATA	ATC
TGA	CAA	TGA	AAT	TTT	TTC	AAA	GGT	GAA	CTG	TGA	AAC	TAG	GGA	CTA	CCT	TTT	GCG	TAT	TAG
Thr	Val	Thr	Leu	Lys	Lys	Phe	Pro	Leu	Asp	Thr	Leu	Ile	Pro	Asp	Gly	Lys	Arg	Ile	Ile>
	250		260		270		280		290		300								
*	*	*	*	*	*	*	*	*	*	*	*								
TGG	GAC	AGT	AGA	AAG	GGC	TTC	ATC	ATA	TCA	AAT	GCA	ACG	TAC	AAA	GAA	ATA	GGG	CTT	CTG
ACC	CTG	TCA	TCT	TTC	CCG	AAG	TAG	TAT	AGT	TTA	CGT	TGC	ATG	TTT	CTT	TAT	CCC	GAA	GAC
Trp	Asp	Ser	Arg	Lys	Gly	Phe	Ile	Ile	Ser	Asn	Ala	Thr	Tyr	Lys	Glu	Ile	Gly	Leu	Leu>
	310		320		330		340		350		360								
*	*	*	*	*	*	*	*	*	*	*	*								
ACC	TGT	GAA	GCA	ACA	GTC	AAT	GGG	CAT	TTG	TAT	AAG	ACA	AAC	TAT	CTC	ACA	CAT	CGA	CAA
TGG	ACA	CTT	CGT	TGT	CAG	TTA	CCC	GTA	AAC	ATA	TTC	TGT	TTG	ATA	GAG	TGT	GTA	GCT	GTT
Thr	Cys	Glu	Ala	Thr	Val	Asn	Gly	His	Leu	Tyr	Lys	Thr	Asn	Tyr	Leu	Thr	His	Arg	Gln>
	370		380		390		400		410		420								
*	*	*	*	*	*	*	*	*	*	*	*								
ACC	AAT	ACA	ATC	ATA	GAT	GTC	CAA	ATA	AGC	ACA	CCA	CGC	CCA	GTC	AAA	TTA	CTT	AGA	GGC
TGG	TTA	TGT	TAG	TAT	CTA	CAG	GTT	TAT	TCG	TGT	GGT	GCG	GGT	CAG	TTT	AAT	GAA	TCT	CCG
Thr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Ser	Thr	Pro	Arg	Pro	Val	Lys	Leu	Leu	Arg	Gly>
	430		440		450		460		470		480								
*	*	*	*	*	*	*	*	*	*	*	*								
CAT	ACT	CTT	GTC	CTC	AAT	TGT	ACT	GCT	ACC	ACT	CCC	TTG	AAC	ACG	AGA	GTT	CAA	ATG	ACC
GTA	TGA	GAA	CAG	GAG	TTA	ACA	TGA	CGA	TGG	TGA	GGG	AAC	TTG	TGC	TCT	CAA	GTT	TAC	TGG
His	Thr	Leu	Val	Leu	Asn	Cys	Thr	Ala	Thr	Thr	Pro	Leu	Asn	Thr	Arg	Val	Gln	Met	Thr>
	490		500		510		520		530		540								
*	*	*	*	*	*	*	*	*	*	*	*								
TGG	AGT	TAC	CCT	GAT	GAA	AAA	AAT	AAG	AGA	GCT	TCC	GTA	AGG	CGA	CGA	ATT	GAC	CAA	AGC
ACC	TCA	ATG	GGA	CTA	CTT	TTT	TTA	TTC	TCT	CGA	AGG	CAT	TCC	GCT	GCT	TAA	CTG	GTT	TCG
Trp	Ser	Tyr	Pro	Asp	Glu	Lys	Asn	Lys	Arg	Ala	Ser	Val	Arg	Arg	Arg	Ile	Asp	Gln	Ser>

SUBSTITUTE SHEET (RULE 26)

23/55

## Fig.15B.

```

      550      560      570      580      590      600
      *      *      *      *      *      *
AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC
TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG
Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp>

      610      620      630      640      650      660
      *      *      *      *      *      *
AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA
TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT
Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser>

      670      680      690      700      710      720
      *      *      *      *      *      *
GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT
Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr>

      730      740      750      760      770      780
      *      *      *      *      *      *
TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA
ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro>

      790      800      810      820      830      840
      *      *      *      *      *      *
AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp>

      850      860      870      880      890      900
      *      *      *      *      *      *
GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT
CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA
Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His>

      910      920      930      940      950      960
      *      *      *      *      *      *
AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC
TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG
Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val>

      970      980      990      1000      1010      1020
      *      *      *      *      *      *
CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC
GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn>

      1030      1040      1050      1060      1070      1080
      *      *      *      *      *      *
AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>

```

SUBSTITUTE SHEET (RULE 26)

24/55

Fig.15C.

1090	1100	1110	1120	1130	1140
* * *	* *	* *	* *	* *	* *
CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG					
GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC					
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu>					
1150	1160	1170	1180	1190	1200
* *	* *	* *	* *	* *	* *
ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG					
TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC					
Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly>					
1210	1220	1230	1240	1250	1260
* *	* *	* *	* *	* *	* *
CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC					
GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG					
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe>					
1270	1280	1290	1300	1310	1320
* *	* *	* *	* *	* *	* *
CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC					
GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG					
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>					
1330	1340	1350	1360	1370	1380
* *	* *	* *	* *	* *	* *
TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG					
AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC					
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro>					
* GGT AAA TGA CCA TTT ACT Gly Lys ***>					

SUBSTITUTE SHEET (RULE 26)

25/55

## Fig.16A.

10	20	30	40	50	60
* * *	* *	* *	* *	* *	* *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC					
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG					
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>					
70	80	90	100	110	120
* * *	* *	* *	* *	* *	* *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG					
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC					
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>					
130	140	150	160	170	180
* * *	* *	* *	* *	* *	* *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA					
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT					
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>					
190	200	210	220	230	240
* * *	* *	* *	* *	* *	* *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC					
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG					
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>					
250	260	270	280	290	300
* * *	* *	* *	* *	* *	* *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC					
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG					
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>					
310	320	330	340	350	360
* * *	* *	* *	* *	* *	* *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA					
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT					
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>					
370	380	390	400	410	420
* * *	* *	* *	* *	* *	* *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT					
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA					
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>					
430	440	450	460	470	480
* * *	* *	* *	* *	* *	* *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT					
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA					
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>					
490	500	510	520	530	540
* * *	* *	* *	* *	* *	* *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT					
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA					
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>					

SUBSTITUTE SHEET (RULE 26)

26/55

Fig.16B.

550	560	570	580	590	600
* * *	* *	* *	* *	* *	* *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA	CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT	Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>			
610	620	630	640	650	660
* * *	* *	* *	* *	* *	* *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT	CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA	Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>			
670	680	690	700	710	720
* * *	* *	* *	* *	* *	* *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC	GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG	Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>			
730	740	750	760	770	780
* * *	* *	* *	* *	* *	* *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG	TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC	Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>			
790	800	810	820	830	840
* * *	* *	* *	* *	* *	* *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AAC GCT TCC GTA AGG CGA	TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TTG CGA AGG CAT TCC GCT	Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Asn Ala Ser Val Arg Arg>			
850	860	870	880	890	900
* * *	* *	* *	* *	* *	* *
CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA	GCT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT	Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>			
910	920	930	940	950	960
* * *	* *	* *	* *	* *	* *
ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA	TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT	Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys>			
970	980	990	1000	1010	1020
* * *	* *	* *	* *	* *	* *
TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT	AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA	Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>			
1030	1040	1050	1060	1070	1080
* * *	* *	* *	* *	* *	* *
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC	CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG	Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>			

SUBSTITUTE SHEET (RULE 26)

27/55

## Fig.16C.

1090	1100	1110	1120	1130	1140
* * *	* *	* *	* *	* *	* *
TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA					
AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT					
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr>					
1150	1160	1170	1180	1190	1200
* * *	* *	* *	* *	* *	* *
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC					
ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG					
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp>					
1210	1220	1230	1240	1250	1260
* * *	* *	* *	* *	* *	* *
GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC					
CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG					
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr>					
1270	1280	1290	1300	1310	1320
* * *	* *	* *	* *	* *	* *
CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG					
GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC					
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys>					
1330	1340	1350	1360	1370	1380
* * *	* *	* *	* *	* *	* *
TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA					
ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT					
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys>					
1390	1400	1410	1420	1430	1440
* * *	* *	* *	* *	* *	* *
GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG					
CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC					
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys>					
1450	1460	1470	1480	1490	1500
* * *	* *	* *	* *	* *	* *
AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG					
TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC					
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>					
1510	1520	1530	1540	1550	1560
* * *	* *	* *	* *	* *	* *
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC					
ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG					
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>					
1570	1580	1590	1600	1610	1620
* * *	* *	* *	* *	* *	* *
GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG					
CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC					
Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>					

SUBSTITUTE SHEET (RULE 26)

28/55

## Fig.16D.

		1630			1640			1650			1660			1670			1680		
	*		*		*		*		*		*		*		*		*		*
AAC	GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC
TTG	CAG	AAG	AGT	ACG	AGG	CAC	TAC	GTA	CTC	CGA	GAC	GTG	TTG	GTG	ATG	TGC	GTC	TTC	TCG
Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser>

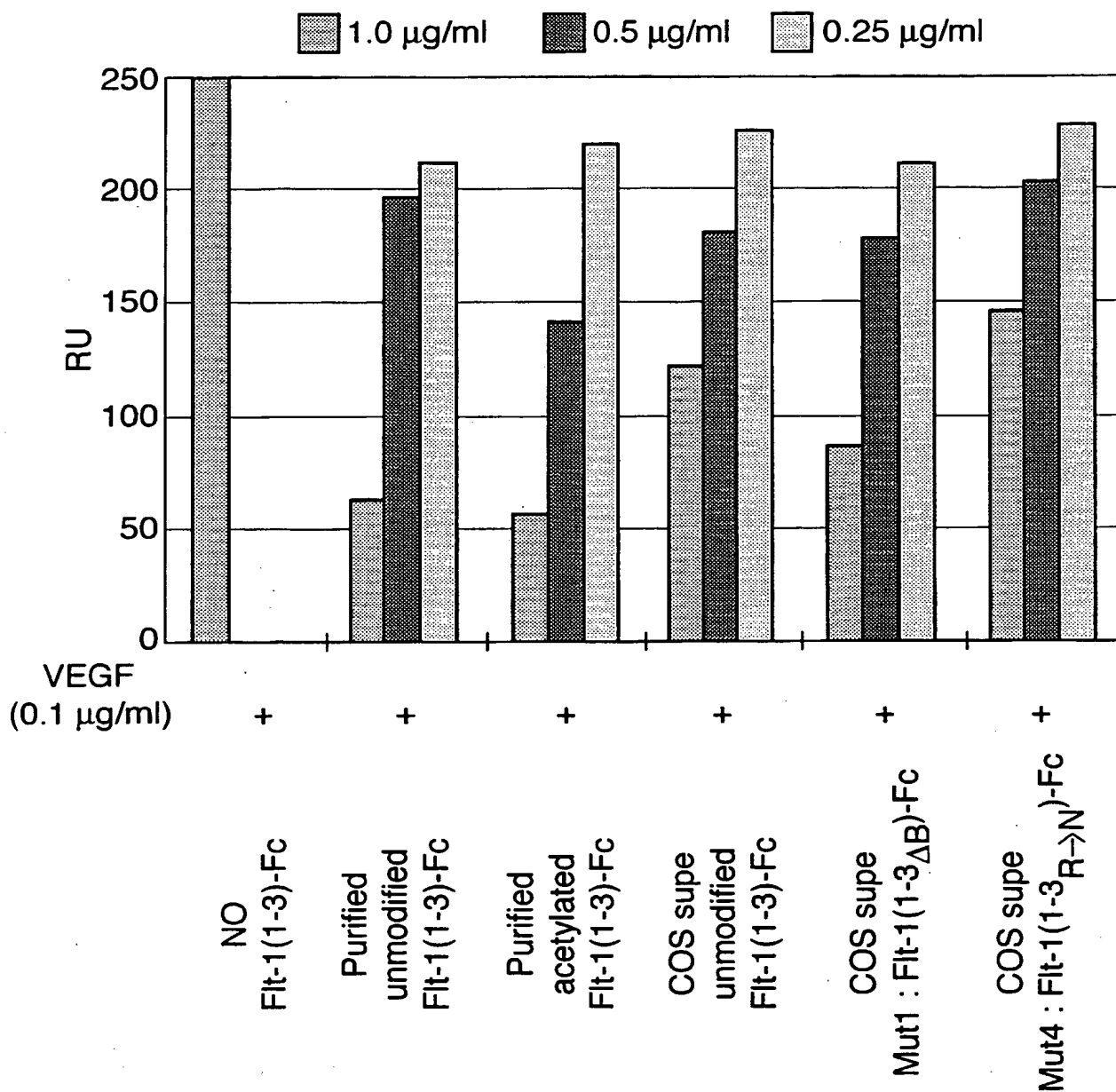
		1690			1700		
	*		*		*		*
CTC	TCC	CTG	TCT	CCG	GGT	AAA	TGA
GAG	AGG	GAC	AGA	GGC	CCA	TTT	ACT
Leu	Ser	Leu	Ser	Pro	Gly	Lys	***>

SUBSTITUTE SHEET (RULE 26)



29/55

Fig.17.



SUBSTITUTE SHEET (RULE 26)

Fig.18.

30/55

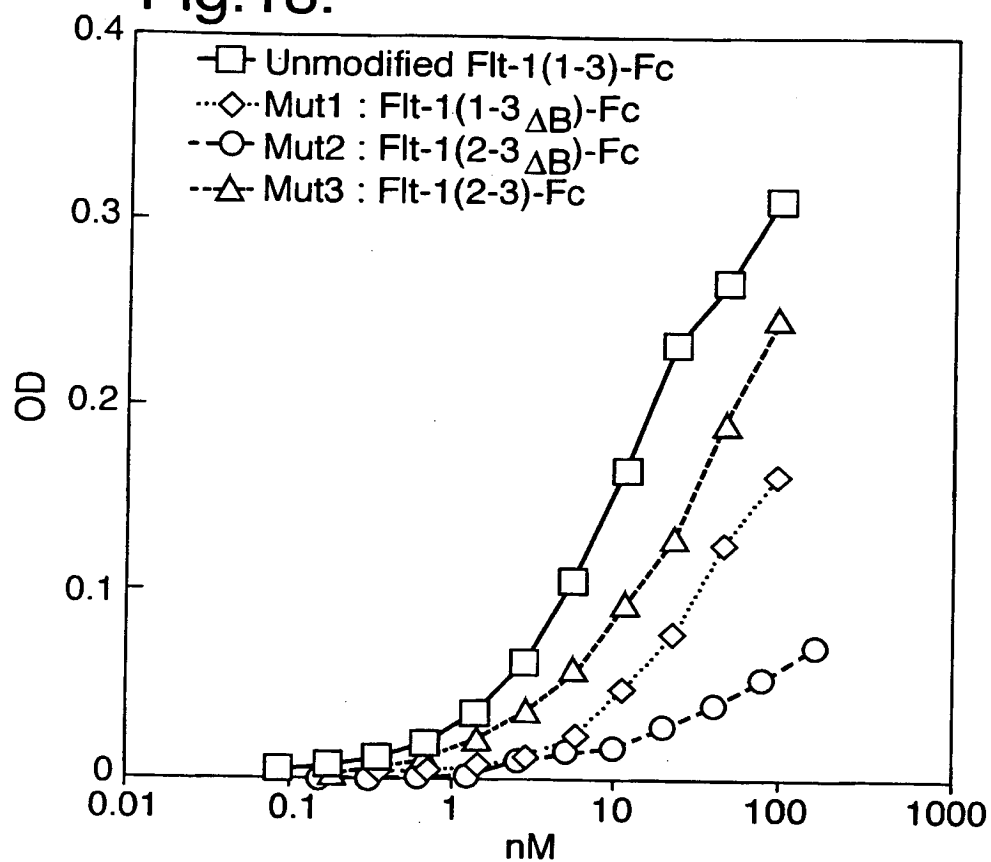
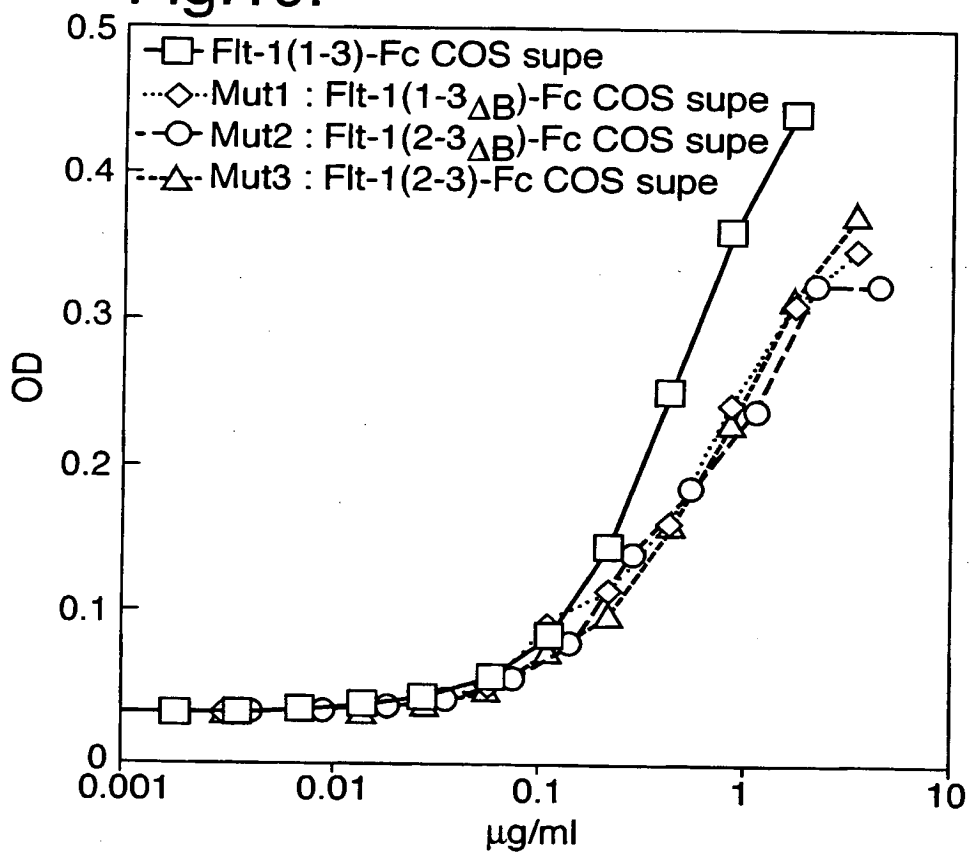


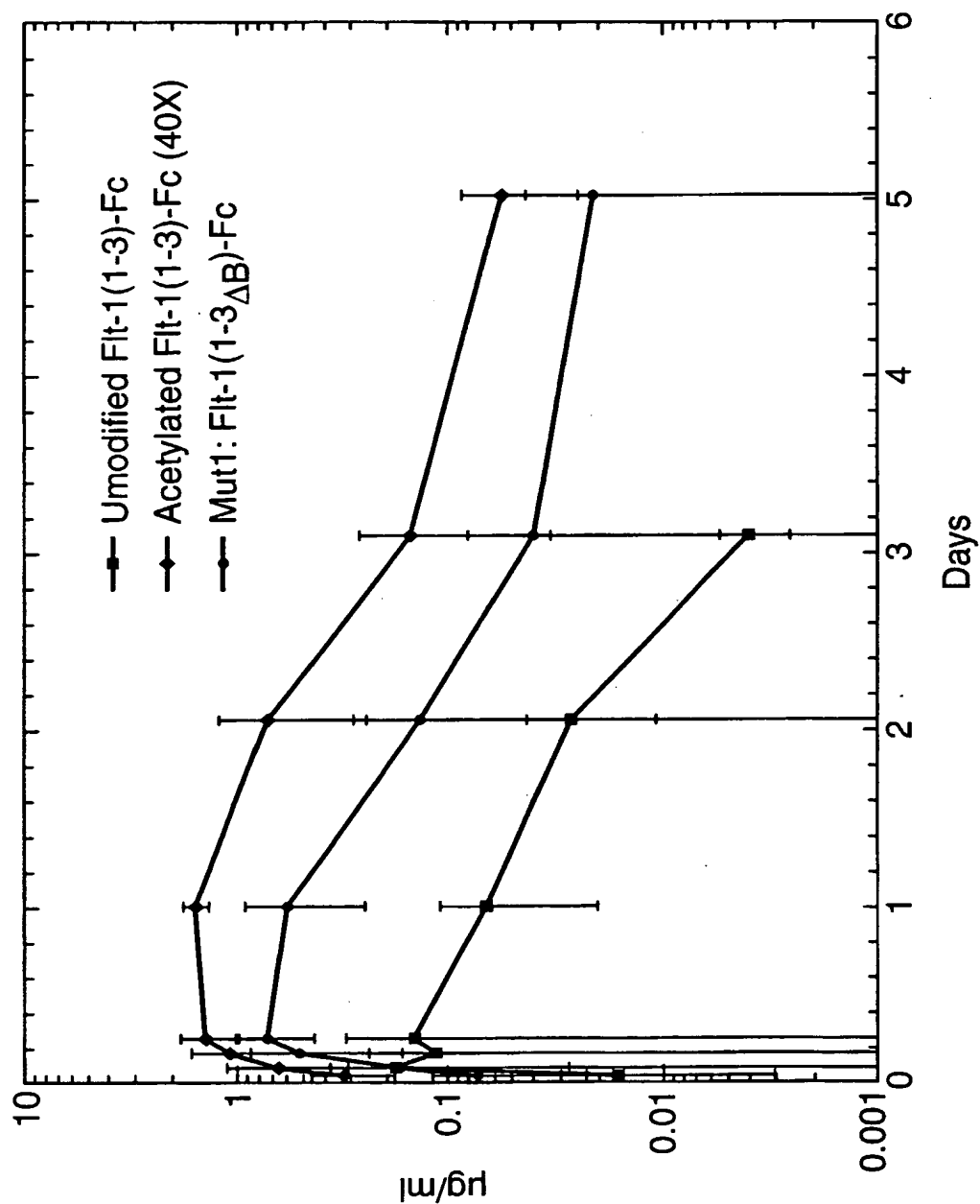
Fig.19.



SUBSTITUTE SHEET (RULE 26)

31/55

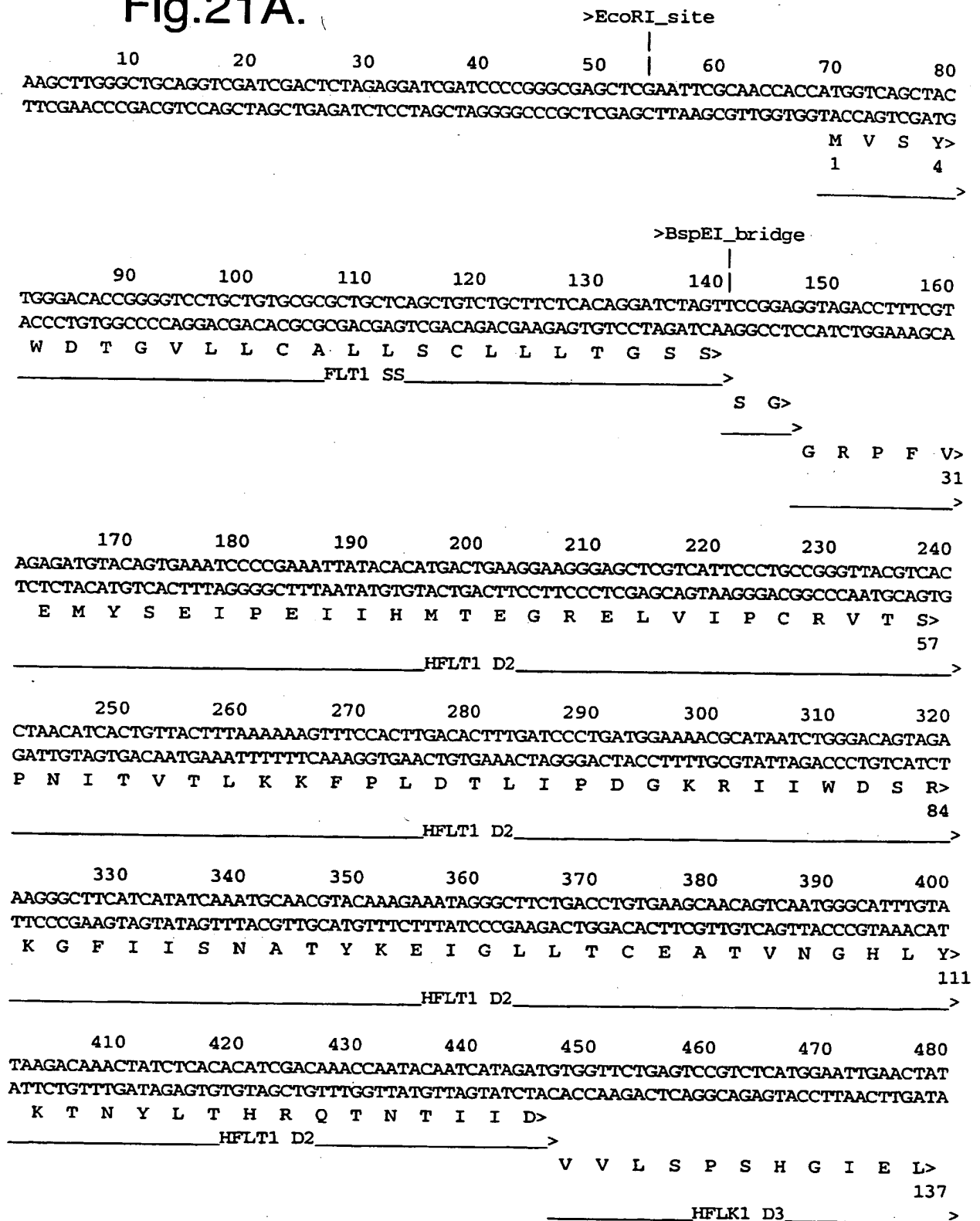
Fig.20.



SUBSTITUTE SHEET (RULE 26)

32/55

Fig.21A.



SUBSTITUTE SHEET (RULE 26)

## Fig.21B.

33/55

490 500 510 520 530 540 550 560  
 CTGTTGGAGAAAAGCTTGTCTTAAATTGTACAGCAAGAACTGAACTAAATGTGGGGATTGACTTCAACTGGGAATACCCCT  
 GACAACCTCTTTTCGAACAGAATTTAACATGTCGTTCTTGACTTGATTTACACCCCTAACTGAAGTTGACCCTTATGGGA  
 S V G E K L V L N C T A R T E L N V G I D F N W E Y P>  
 164  
 \_\_\_\_\_HFLK1 D3\_\_\_\_\_>

570 580 590 600 610 620 630 640  
 TCTTCGAAGCATCAGCATAAGAACTTGTAACCGAGACCTAAAAACCCAGTCTGGGAGTGAGATGAAGAAATTTTGTAG  
 AGAAGCTTCGTAGTCGTATTCTTTGAACATTTGGCTCTGGATTTTGGGTCAGACCCTCACTCTACTTCTTTAAAAACTC  
 S S K H Q H K K L V N R D L K T Q S G S E M K K F L S>  
 191  
 \_\_\_\_\_HFLK1 D3\_\_\_\_\_>

650 660 670 680 690 700 710 720  
 CACCTTAACCTATAGATGGTGTAAACCGGAGTGACCAAGGATTTGTACACCTGTGCAGCATCCAGTGGGCTGATGACCAAGA  
 GTGGAATTGATATCTACCACATTGGGCCTCACTGGTTCTTAACATGTGGACACGTCGTAGGTCACCCGACTACTGGTTCT  
 T L T I D G V T R S D Q G L Y T C A A S S G L M T K>  
 217  
 \_\_\_\_\_HFLK1 D3\_\_\_\_\_>

>Srf\_Bridge\_  
 |  
 730 740 750 760 770 780 790 800  
 AGAACAGCACATTTGTTCAGGGTCCATGAAAAGGGCCCGGGCGACAAAACCTCACACATGCCCACCGTGCCCAGCACCTGAA  
 TCTTGTCTGTAAACAGTCCCAGGTACTTTTCCCGGGCCCGCTGTTTGTAGTGTGTACGGGTGGCACGGGTCTGTGGACTT  
 K N S T F V R V H E K>  
 \_\_\_\_\_HFLK1 D3\_\_\_\_\_>  
 G P G>  
 \_\_\_\_\_>  
 D K T H T C P P C P A P E>  
 244  
 \_\_\_\_\_FCΔC1 (A)\_\_\_\_\_>

810 820 830 840 850 860 870 880  
 CTCCTGGGGGGACCGTCAGTCTTCTCTTCCCCCAAACCCAAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTACAC  
 GAGGACCCCTTGGCAGTCAGAAGGAGAAGGGGGGTTTGGGTCTCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTG  
 L L G G P S V F L F P P K P K D T L M I S R T P E V T>  
 271  
 \_\_\_\_\_FCΔC1 (A)\_\_\_\_\_>

890 900 910 920 930 940 950 960  
 ATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG  
 TACGCACCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTAC  
 C V V V D V S H E D P E V K F N W Y V D G V E V H N>  
 297  
 \_\_\_\_\_FCΔC1 (A)\_\_\_\_\_>

970 980 990 1000 1010 1020 1030 1040  
 CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTTCAGCGTCTCACCCTGTCACCAGGACTGG  
 GGTTCGTGTTTCGGCGCCCTCCTCGTCATGTTGTCTGTCATGGCACACCAAGTTCGAGGAGTGGCAGGACGTGGTCTGACC  
 A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W>  
 324  
 \_\_\_\_\_FCΔC1 (A)\_\_\_\_\_>

SUBSTITUTE SHEET (RULE 26)

34/55

## Fig.21C.

1050 1060 1070 1080 1090 1100 1110 1120  
CTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAA  
GACTTACCGTTCCTCATGTTCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTT  
L N G K E Y K C K V S N K A L P A P I E K T I S K A K>  
351  
FCAC1 (A) >

>A>C\_A\_allotype  
|  
>G>T\_A\_allotype  
| |  
1130 1140 1150 1160 1170 1180 1190 1200  
AGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT  
TCCCGTCGGGGCTCTTGGTGTCACATGTGGGACGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGA  
G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T>  
377  
FCAC1 (A) >

1210 1220 1230 1240 1250 1260 1270 1280  
GCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACC  
CGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCCTCTCGTTACCCGTCGGCCTCTTGTGTATGTTCTGTG  
C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T>  
404  
FCAC1 (A) >

>T>C  
|  
1290 1300 1310 1320 1330 1340 1350 1360  
ACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGG  
TGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGATATCGTTTCGAGTGGCACCTGTCTCGTCCACCGTCGTCCC  
T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G>  
431  
FCAC1 (A) >

1370 1380 1390 1400 1410 1420 1430 1440  
GAACGTCTTCTCATGTCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA  
CTTGACAGAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCAT  
N V F S C S V M H E A L H N H Y T Q K S L S L S P G>  
457  
FCAC1 (A) >

>NotI\_site  
|  
|1450  
AATGAGCGGCCGC  
TTACTCGCCGGCG  
K \*>  
458  
>

SUBSTITUTE SHEET (RULE 26)

35/55

Fig.22A.

>EcoRI\_site

10 20 30 40 50 60 70 80

AAGCTTGGGCTGCAGGTCGATCGACTCTAGAGGATCGATCCCCGGGCGAGCTCGAATTCGCAACCACCATGGTCAGCTAC  
TTCGAACCCGACGTCCAGCTAGCTGAGATCTCCTAGCTAGGGGCCCCGCTCGAGCTTAAGCGTTGGTGGTACCAGTCGATG

M V S Y>  
1 4

>BspEI\_bridge

90 100 110 120 130 140 150 160

TGGGACACCCGGGTCCTGCTGTGCGCGCTGCTCAGCTGTCTGCTTCTCACAGGATCTAGTTCCGGAGGTAGACCTTTTCGT  
ACCTGTGGCCCCAGGACGACACGCGCGACGAGTCGACAGACGAAGAGTGTCTAGATCAAGGCCTCCATCTGGAAAGCA  
W D T G V L L C A L L S C L L L T G S S>

\_\_\_\_\_FLT1 SIGNAL SEQUENCE\_\_\_\_\_

S G>

G R P F V>  
31

170 180 190 200 210 220 230 240

AGAGATGTACAGTGAATCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTACGTCAC  
TCTCTACATGTCACCTTTAGGGGCTTTAATATGTGTACTGACTTCCTTCCCTCGAGCAGTAAGGGACGGCCCAATGCAGTG  
E M Y S E I P E I I H M T E G R E L V I P C R V T S>

\_\_\_\_\_FLT1 IG DOMAIN 2\_\_\_\_\_

250 260 270 280 290 300 310 320

CTAACATCACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTTGATCCCTGATGGAAAACGCATAATCTGGGACAGTAGA  
GATTGTAGTGACAATGAAATTTTTTCAAAGGTGAACGTGAAACTAGGGACTACCTTTTGGCGTATTAGACCCTGTCATCT  
P N I T V T L K K F P L D T L I P D G K R I I W D S R>

\_\_\_\_\_FLT1 IG DOMAIN 2\_\_\_\_\_

330 340 350 360 370 380 390 400

AAGGGCTTCATCATATCAAATGCAACGTACAAAGAAATAGGGCTTCCTGACCTGTGAAGCAACAGTCAATGGGCATTGTGTA  
TTCCCGAAGTAGTATAGTTTACGTTGCATGTTTCTTTATCCCGAAGACTGGACACTTCGTTGTCAGTTACCCGTAACAT  
K G F I I S N A T Y K E I G L L T C E A T V N G H L Y>

\_\_\_\_\_FLT1 IG DOMAIN 2\_\_\_\_\_

410 420 430 440 450 460 470 480

TAAGACAAACTATCTCACACATCGACAAACCAATACAATCATAGATATCCAGCTGTTGCCAGGAAGTCGCTGGAGCTGC  
ATTCTGTTTGATAGAGTGTGTAGCTGTTTGGTTATGTTAGTATCTATAGGTCGACAAACGGGTCCTTCAGCGACCTCGACG  
K T N Y L T H R Q T N T I I D>

\_\_\_\_\_FLT1 IG DOMAIN 2\_\_\_\_\_

I Q L L P R K S L E L>  
137

\_\_\_\_\_VEGFR3 (FLT4) IG DOMAIN 3\_\_\_\_\_

SUBSTITUTE SHEET (RULE 26)

36/55

## Fig.22B.

490 500 510 520 530 540 550 560  
 TGGTAGGGGAGAAGCTGGTCCTCAACTGCACCGTGTGGGCTGAGTTTAACTCAGGTGTCACCTTTGACTGGGACTACCCA  
 ACCATCCCCCTCTTCGACCAGGAGTTGACGTGGCACACCCGACTCAAATTGAGTCCACAGTGGAAGTACCCTGATGGGT  
 L V G E K L V L N C T V W A E F N S G V T F D W D Y P>  
 164  
 \_\_\_\_\_VEGFR3 (FLT4) IG DOMAIN 3\_\_\_\_\_  
 570 580 590 600 610 620 630 640  
 GGGGAAGCAGGCAGAGCGGGGTAAGTGGGTGCCCCGAGCGACGCTCCCAACAGACCCACACAGAAGTCTCCAGCATCCTGAC  
 CCCTTCGTCCGTCTCGCCCCATTACCCACGGGCTCGCTGCGAGGGTTGTCTGGGTGTGTCTTGAGAGGTCTGAGGACTG  
 G K Q A E R G K W V P E R R S Q Q T H T E L S S I L T>  
 191  
 \_\_\_\_\_VEGFR3 (FLT4) IG DOMAIN 3\_\_\_\_\_  
 650 660 670 680 690 700 710 720  
 CATCCACAACGTCAGCCAGCACGACCTGGGCTCGTATGTGTGCAAGGCCAACACGGCATCCAGCGATTTCGGGAGAGCA  
 GTAGGTGTTGCAGTCGGTCTGTGCTGGACCCGAGCATAACACAGTTCGGGTGTTGTCGGTAGGTCTGCTAAAGCCCTCTCGT  
 I H N V S Q H D L G S Y V C K A N N G I Q R F R E S>  
 217  
 \_\_\_\_\_VEGFR3 (FLT4) IG DOMAIN 3\_\_\_\_\_  
 730 740 750 760 770 780 790 800  
 CCGAGGTCAATTGTGCATGAAAAATGGCCCGGGCGACAAAATCACACATGCCCCACCGTGCCCGAGCACCTGAATCCTGGGG  
 GGCTCCAGTAACACGTACTTTTACCGGGCCCCGCTGTTTGTAGTGTGTACGGGTGGCACGGGTCTGGACTTGAGGACCCC  
 T E V I V H E N>  
 \_\_\_\_\_VEGFR3 (FLT4) IG\_\_\_\_\_  
 G P G>  
 \_\_\_\_\_>  
 D K T H T C P P C P A P E L L G>  
 244  
 \_\_\_\_\_FCAC1 - A ALLOTYP E\_\_\_\_\_  
 810 820 830 840 850 860 870 880  
 GGACCGTCAGTCTTCCTCTTCCCCCAAAACCAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGT  
 CCTGGCAGTCAGAAGGAGAAGGGGGGTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCACCA  
 G P S V F L F P P K P K D T L M I S R T P E V T C V V>  
 271  
 \_\_\_\_\_FCAC1 - A ALLOTYP E\_\_\_\_\_  
 890 900 910 920 930 940 950 960  
 GGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAA  
 CCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTT  
 V D V S H E D P E V K F N W Y V D G V E V H N A K T>  
 297  
 \_\_\_\_\_FCAC1 - A ALLOTYP E\_\_\_\_\_  
 970 980 990 1000 1010 1020 1030 1040  
 AGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCTCACCCTCCTGCACCAGGACTGGCTGAATGGC  
 TCGGCGCCCTCCTCGTCATGTTGTCTGTGCATGGCACACCAGTCCGAGGAGTGGCAGGACGTGGTCTGACCGACTTACCG  
 K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G>  
 324  
 \_\_\_\_\_FCAC1 - A ALLOTYP E\_\_\_\_\_  
 \_\_\_\_\_>

SUBSTITUTE SHEET (RULE 26)



37/55

## Fig.22C.

1050 1060 1070 1080 1090 1100 1110 1120  
AAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCC  
TTCCTCATGTTACAGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTTTCCCGTCGG  
K E Y K C K V S N K A L P A P I E K T I S K A K G Q P>  
351  
\_\_\_\_FCAC1 - A ALLOTYPE\_\_\_\_>

>A>C\_A\_allotype  
|  
>G>T\_A\_allotype  
| | |  
1130 1140 1150 1160 1170 1180 1190 1200  
CCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCA  
GGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAGT  
R E P Q V Y T L P P S R D E L T K N Q V S L T C L V>  
377  
\_\_\_\_FCAC1 - A ALLOTYPE\_\_\_\_>

1210 1220 1230 1240 1250 1260 1270 1280  
AAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCC  
TTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCTCTCGTTACCCGTCGGCCTCTTGTGTGATGTTCTGGTGGGAGGG  
K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P>  
404  
\_\_\_\_FCAC1 - A ALLOTYPE\_\_\_\_>

>T>C  
|  
1290 1300 1310 1320 1330 1340 1350 1360  
GTGCTGGACTCCGACGGCTCCTTCTTCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTT  
CACGACCTGAGGCTGCCGAGGAAGAAGGAGATATCGTTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCTTGCAGAA  
V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F>  
431  
\_\_\_\_FCAC1 - A ALLOTYPE\_\_\_\_>

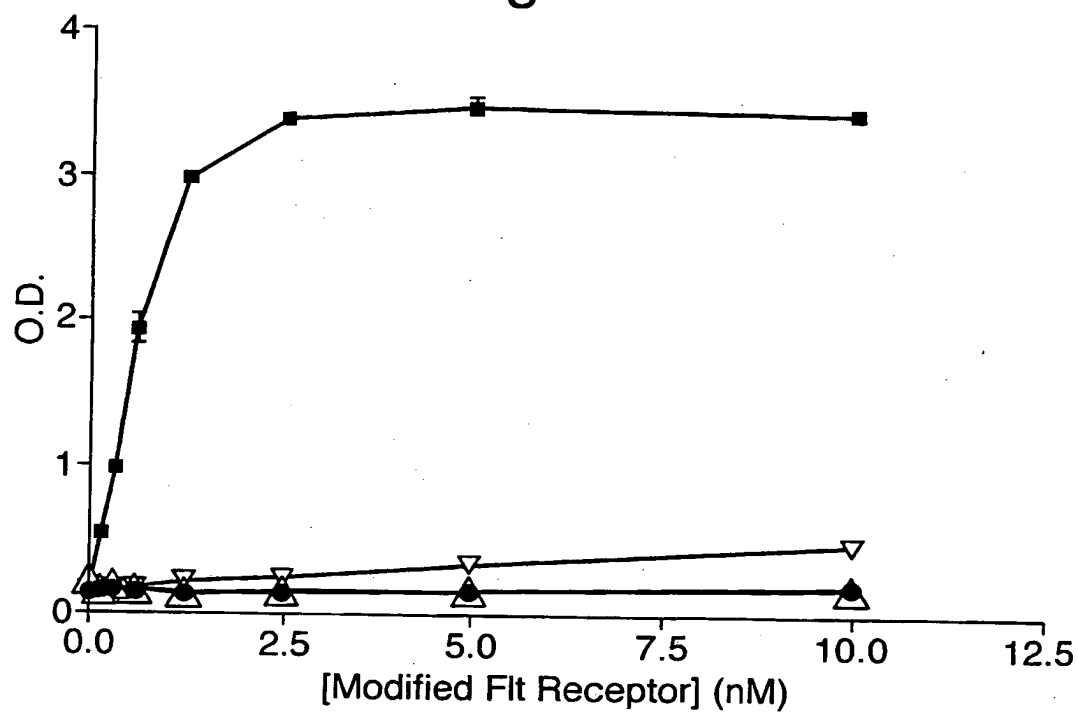
>NotI\_site  
|  
1370 1380 1390 1400 1410 1420 1430 1440  
CTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAAGAGCCTCTCCCTGTC'TCCGGGTAAATGAGCGG  
GAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGTATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTACTCGCC  
S C S V M H E A L H N H Y T Q K S L S L S P G K \*>  
455  
\_\_\_\_FCAC1 - A ALLOTYPE\_\_\_\_>

CCGC  
GGCG

SUBSTITUTE SHEET (RULE 26)

38/55

Fig.23.



- Flt1D2Flk1D3.FcdeltaC1(a)
- △ Flt1D2VEGFR3D3.FcdeltaC1(a)
- ▽ TIE2-Fc
- Flt1(1-3)-Fc

SUBSTITUTE SHEET (RULE 26)

39/55

Fig.24A.

```

      10      20      30      40      50      60
      *      *      *      *      *      *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
M V S Y W D T G V L L C A L L S C L L L>
1_____5_____hFLT1 SIGNAL SEQUENCE_____15_____20>

      70      80      90      100      110      120
      *      *      *      *      *      *
ACA GGA TCT AGT TCC GGA AGT GAT ACC GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC
TGT CCT AGA TCA AGG CCT TCA CTA TGG CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG
T G S S S G>
21_hFLT1 SIGNAL SEQ_26>

      S D T G R P F V E M Y S E I>
     27_____30_____hFLT1 IG DOMAIN 2_____40>

     130      140      150      160      170      180
     *      *      *      *      *      *
CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA
GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT
P E I I H M T E G R E L V I P C R V T S>
41_____45_____hFLT1 IG DOMAIN 2_____55_____60>

     190      200      210      220      230      240
     *      *      *      *      *      *
CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA
GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT
P N I T V T L K K F P L D T L I P D G K>
61_____65_____hFLT1 IG DOMAIN 2_____75_____80>

     250      260      270      280      290      300
     *      *      *      *      *      *
CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA
GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT
R I I W D S R K G F I I S N A T Y K E I>
81_____85_____hFLT1 IG DOMAIN 2_____95_____100>

     310      320      330      340      350      360
     *      *      *      *      *      *
GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA
CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT
G L L T C E A T V N G H L Y K T N Y L T>
101_____105_____hFLT1 IG DOMAIN 2_____115_____120>

     370      380      390      400      410      420
     *      *      *      *      *      *
CAT CGA CAA ACC AAT ACA ATC ATA GAT GTG GTT CTG AGT CCG TCT CAT GGA ATT GAA CTA
GTA GCT GTT TGG TTA TGT TAG TAT CTA CAC CAA GAC TCA GGC AGA GTA CCT TAA CTT GAT
H R Q T N T I I D>
121_____hFLT1 IG DOMAIN 2_____129>

      V V L S P S H G I E L>
     130_____hFLK1 IG DOMAIN 3_____140>

```

SUBSTITUTE SHEET (RULE 26)

## Fig.24B.

40/55

```

      430      440      450      460      470      480
      *      *      *      *      *      *
TCT GTT GGA GAA AAG CTT GTC TTA AAT TGT ACA GCA AGA ACT GAA CTA AAT GTG GGG ATT
AGA CAA CCT CTT TTC GAA CAG AAT TTA ACA TGT CGT TCT TGA CTT GAT TTA CAC CCC TAA
S V G E K L V L N C T A R T E L N V G I>
141_____145_____hFLK1 IG DOMAIN 3_____155_____160>

      490      500      510      520      530      540
      *      *      *      *      *      *
GAC TTC AAC TGG GAA TAC CCT TCT TCG AAG CAT CAG CAT AAG AAA CTT GTA AAC CGA GAC
CTG AAG TTG ACC CTT ATG GGA AGA AGC TTC GTA GTC GTA TTC TTT GAA CAT TTG GCT CTG
D F N W E Y P S S K H Q H K K L V N R D>
161_____165_____hFLK1 IG DOMAIN 3_____175_____180>

      550      560      570      580      590      600
      *      *      *      *      *      *
CTA AAA ACC CAG TCT GGG AGT GAG ATG AAG AAA TTT TTG AGC ACC TTA ACT ATA GAT GGT
GAT TTT TGG GTC AGA CCC TCA CTC TAC TTC TTT AAA AAC TCG TGG AAT TGA TAT CTA CCA
L K T Q S G S E M K K F L S T L T I D G>
181_____185_____hFLK1 IG DOMAIN 3_____195_____200>

      610      620      630      640      650      660
      *      *      *      *      *      *
GTA ACC CGG AGT GAC CAA GGA TTG TAC ACC TGT GCA GCA TCC AGT GGG CTG ATG ACC AAG
CAT TGG GCC TCA CTG GTT CCT AAC ATG TGG ACA CGT CGT AGG TCA CCC GAC TAC TGG TTC
V T R S D Q G L Y T C A A S S G L M T K>
201_____205_____hFLK1 IG DOMAIN 3_____215_____220>

      670      680      690      700      710      720
      *      *      *      *      *      *
AAG AAC AGC ACA TTT GTC AGG GTC CAT GAA AAG GAC AAA ACT CAC ACA TGC CCA CCG TGC
TTC TTG TCG TGT AAA CAG TCC CAG GTA CTT TTC CTG TTT TGA GTG TGT ACG GGT GGC ACG
K N S T F V R V H E K>
221_____hFLK1 IG DOMAIN 3_____231>

      D K T H T C P P C>
      232_____hFCAC1 A_____240>

      730      740      750      760      770      780
      *      *      *      *      *      *
CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC
GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG
P A P E L L G G P S V F L F P P K P K D>
241_____245_____hFCAC1 A_____255_____260>

      790      800      810      820      830      840
      *      *      *      *      *      *
ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA
TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT
T L M I S R T P E V T C V V V D V S H E>
261_____265_____hFCAC1 A_____275_____280>

      850      860      870      880      890      900
      *      *      *      *      *      *
GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA
CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CCG TTC TGT
D P E V K F N W Y V D G V E V H N A K T>
281_____285_____hFCAC1 A_____295_____300>

```

SUBSTITUTE SHEET (RULE 26)

41/55

## Fig.24C.

910	920	930	940	950	960
*	*	*	*	*	*
AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG					
TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC					
K P R E E Q Y N S T Y R V V S V L T V L>					
301_____305_____hFCAC1 A _____315_____320>					
970	980	990	1000	1010	1020
*	*	*	*	*	*
CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA					
GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT					
H Q D W L N G K E Y K C K V S N K A L P>					
321_____325_____hFCAC1 A _____335_____340>					
1030	1040	1050	1060	1070	1080
*	*	*	*	*	*
GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC					
CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG					
A P I E K T I S K A K G Q P R E P Q V Y>					
341_____345_____hFCAC1 A _____355_____360>					
1090	1100	1110	1120	1130	1140
*	*	*	*	*	*
ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC					
TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG					
T L P P S R D E L T K N Q V S L T C L V>					
361_____365_____hFCAC1 A _____375_____380>					
1150	1160	1170	1180	1190	1200
*	*	*	*	*	*
AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC					
TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG					
K G F Y P S D I A V E W E S N G Q P E N>					
381_____385_____hFCAC1 A _____395_____400>					
1210	1220	1230	1240	1250	1260
*	*	*	*	*	*
AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG					
TTG ATG TTC TGG TGC GGA GGC CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC					
N Y K T T P P V L D S D G S F F L Y S K>					
401_____405_____hFCAC1 A _____415_____420>					
1270	1280	1290	1300	1310	1320
*	*	*	*	*	*
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT					
GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA					
L T V D K S R W Q G N V F S C S V M H>					
421_____425_____hFCAC1 A _____435_____440>					
1330	1340	1350	1360	1370	
*	*	*	*	*	
GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA					
CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT					
E A L H N H Y T Q K S L S L S P G K *>					
441_____445_____hFCAC1 A _____455_____458>					

SUBSTITUTE SHEET (RULE 26)

42/55

Fig.25A.

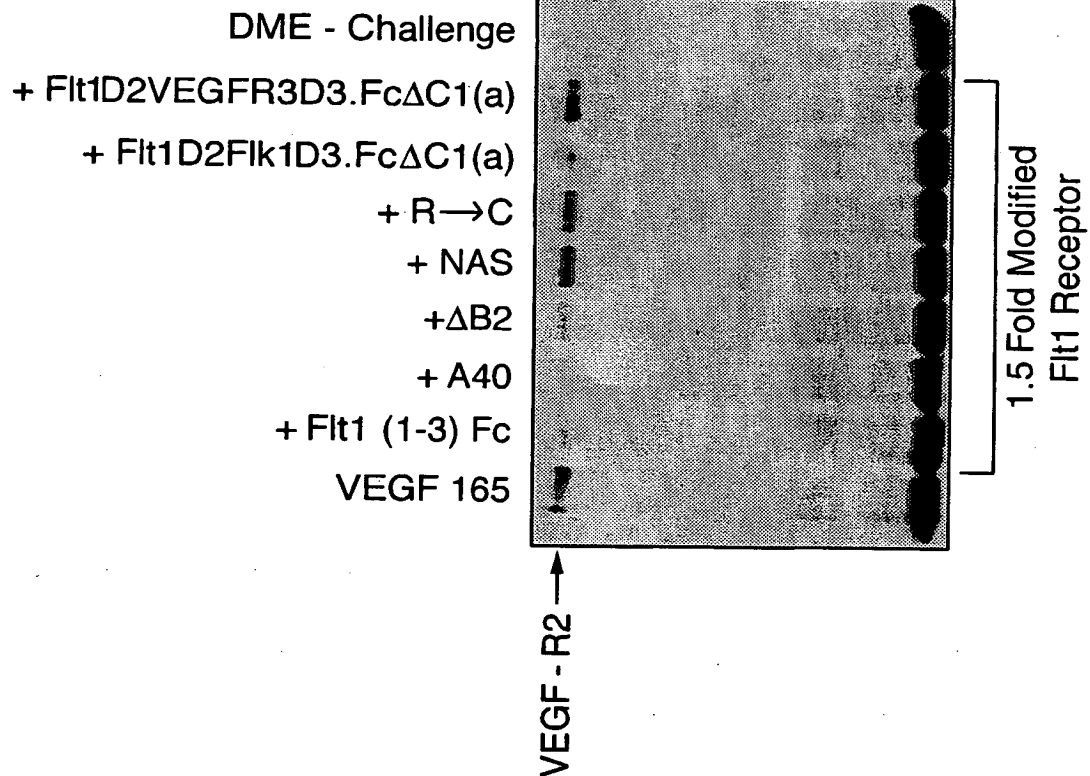
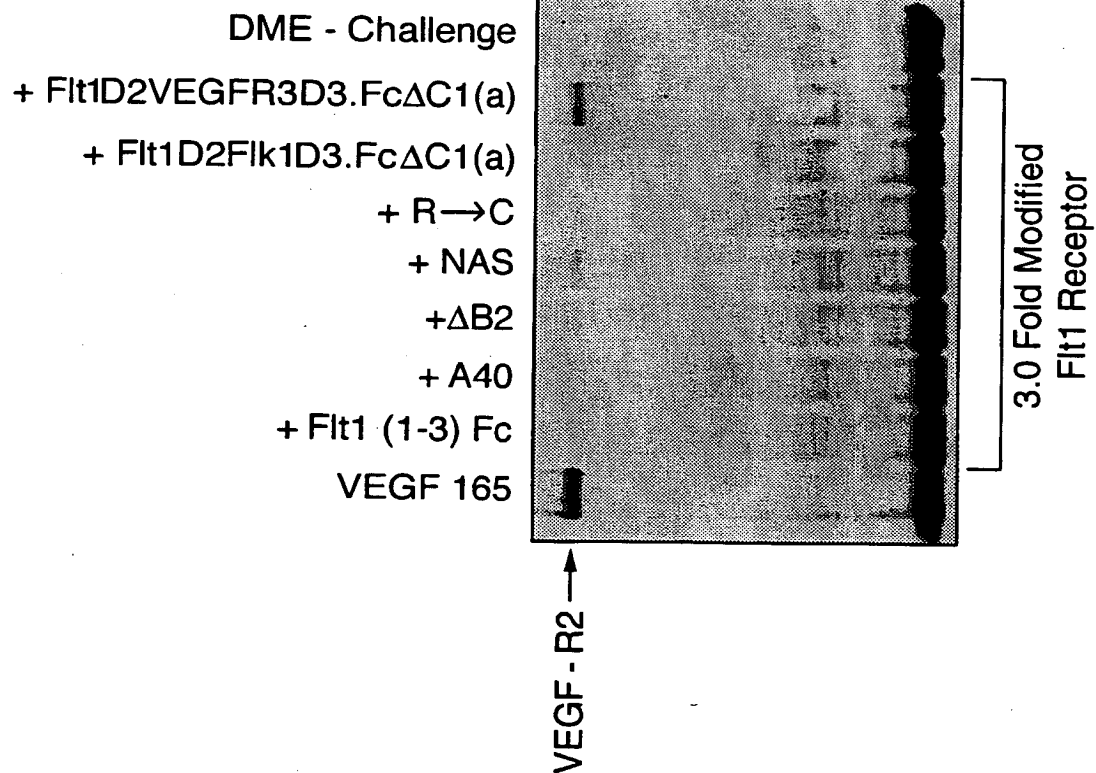


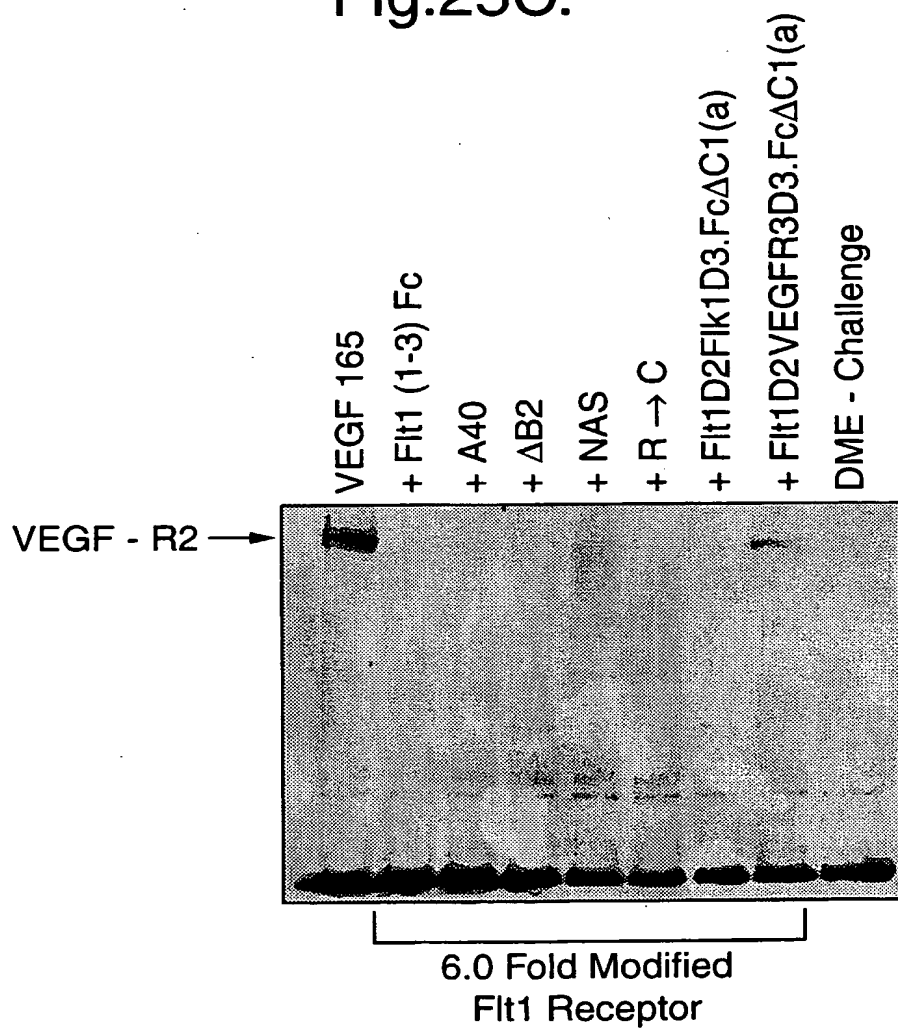
Fig.25B.



SUBSTITUTE SHEET (RULE 26)

43/55

Fig.25C.



SUBSTITUTE SHEET (RULE 26)

Fig.26A.

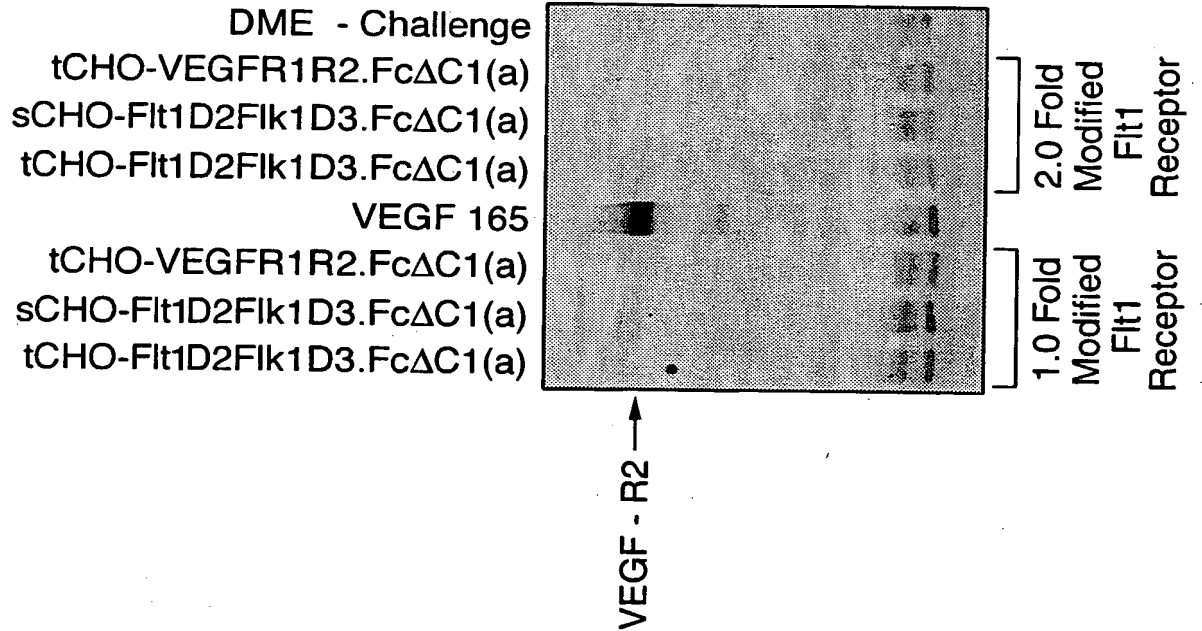
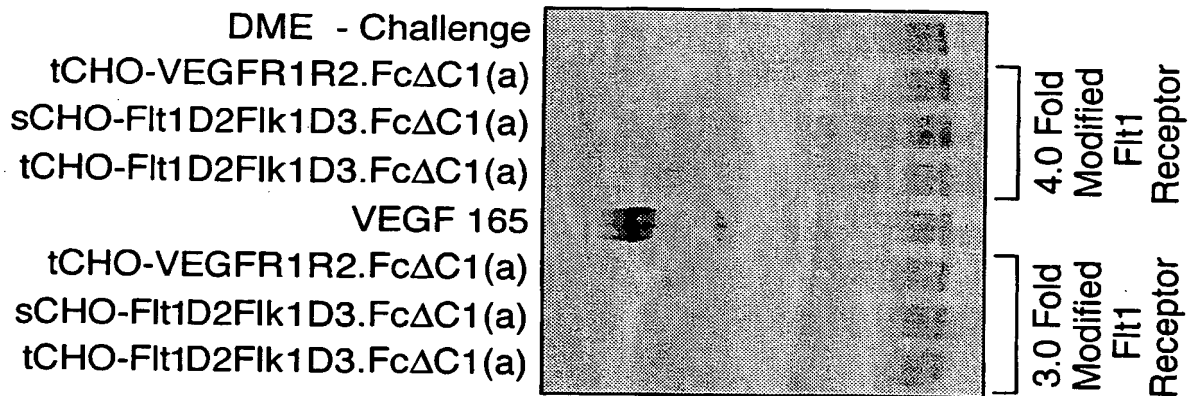


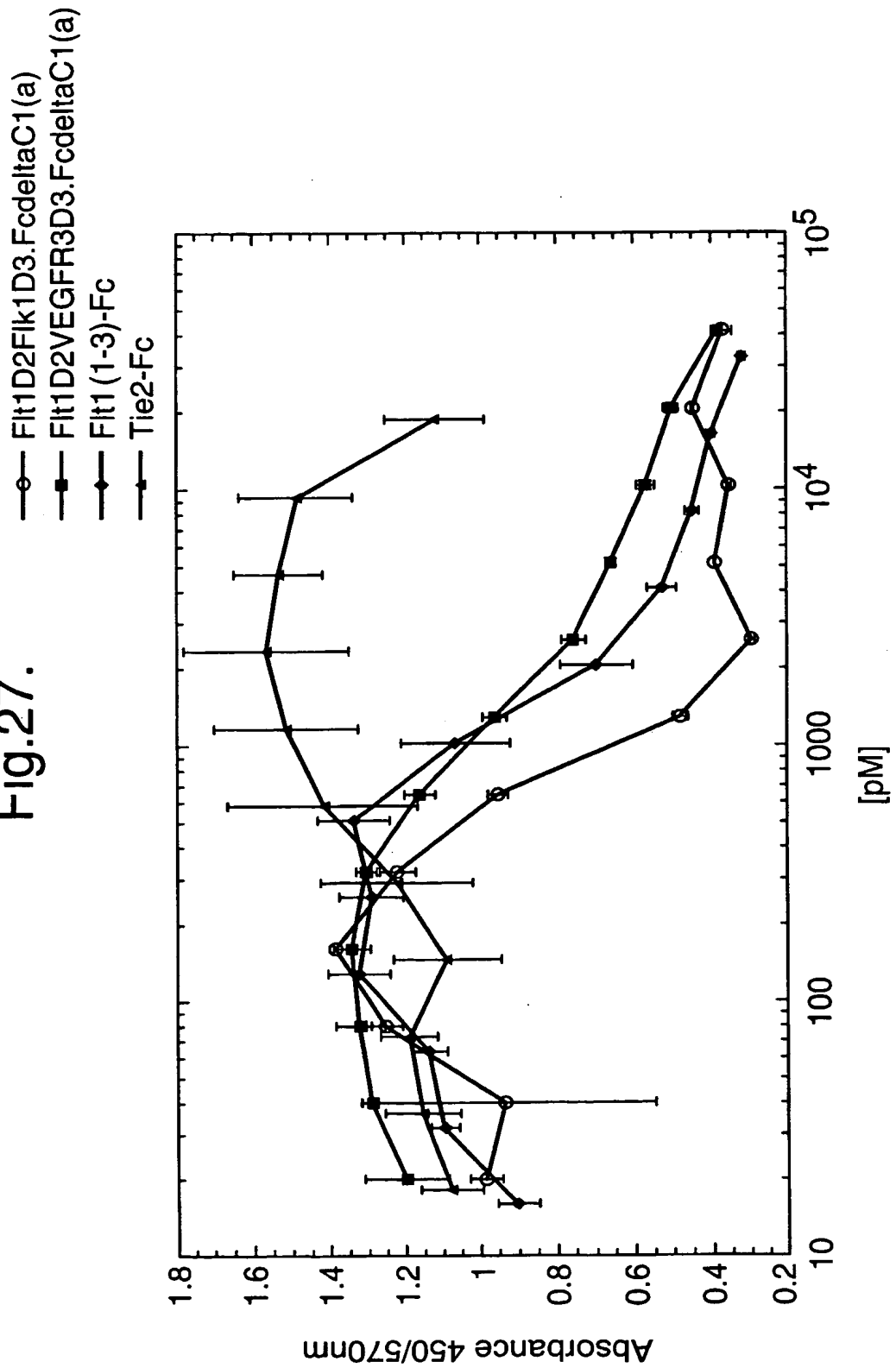
Fig.26B.





45/55

Fig.27.



SUBSTITUTE SHEET (RULE 26)

46/55

Fig.28.

Binding Stoichiometry of hVEGF165 to Flt1D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)		
hVEGF165 (nM)	VEGF/Flt1D2Flk1D3.FcΔC1(a)	VEGF/VEGFR1R2-FcΔC1(a)
1	0.93	0.98
10	0.97	0.94
50	1	0.99
Average ± StDev	0.96 ± 0.03	0.97 ± 0.02

SUBSTITUTE SHEET (RULE 26)

47/55

Fig.29.

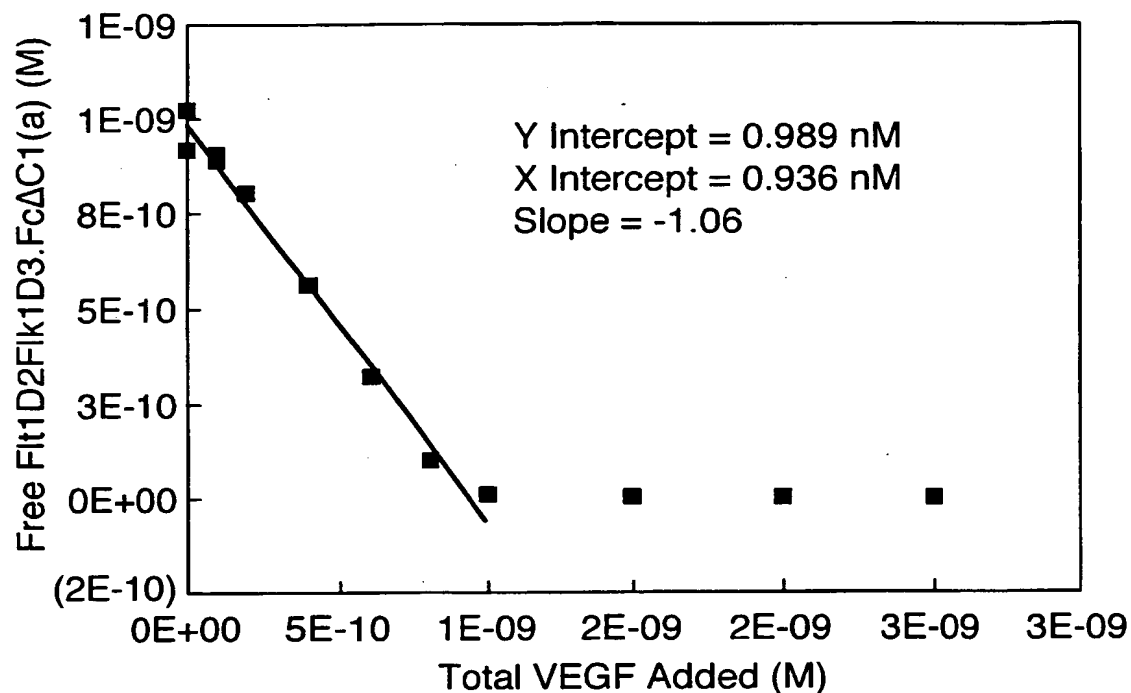
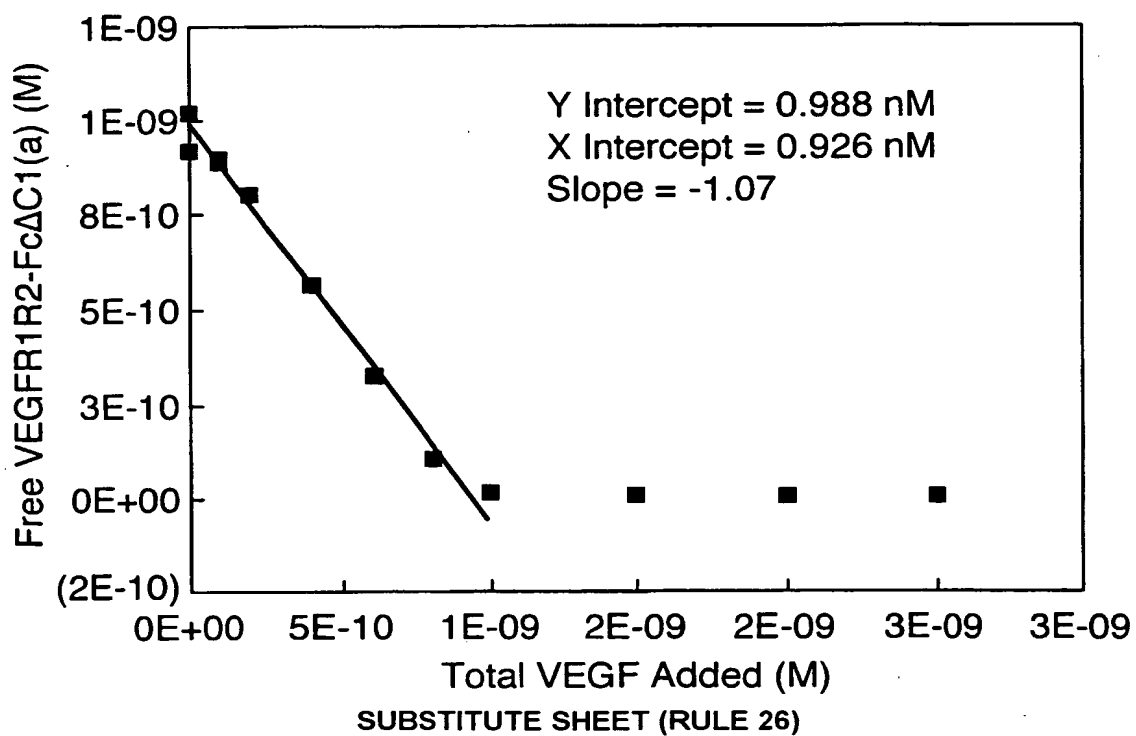
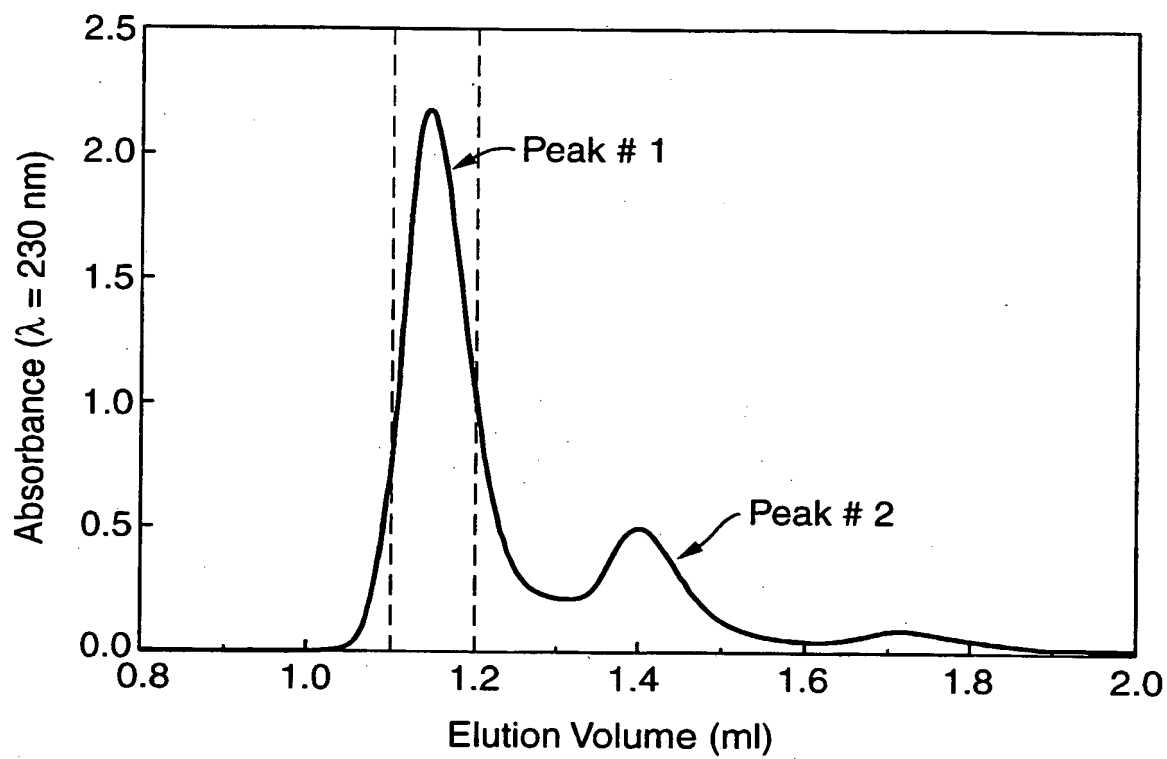


Fig.30.



48/55

Fig.31.



SUBSTITUTE SHEET (RULE 26)

49/55

Fig.32.

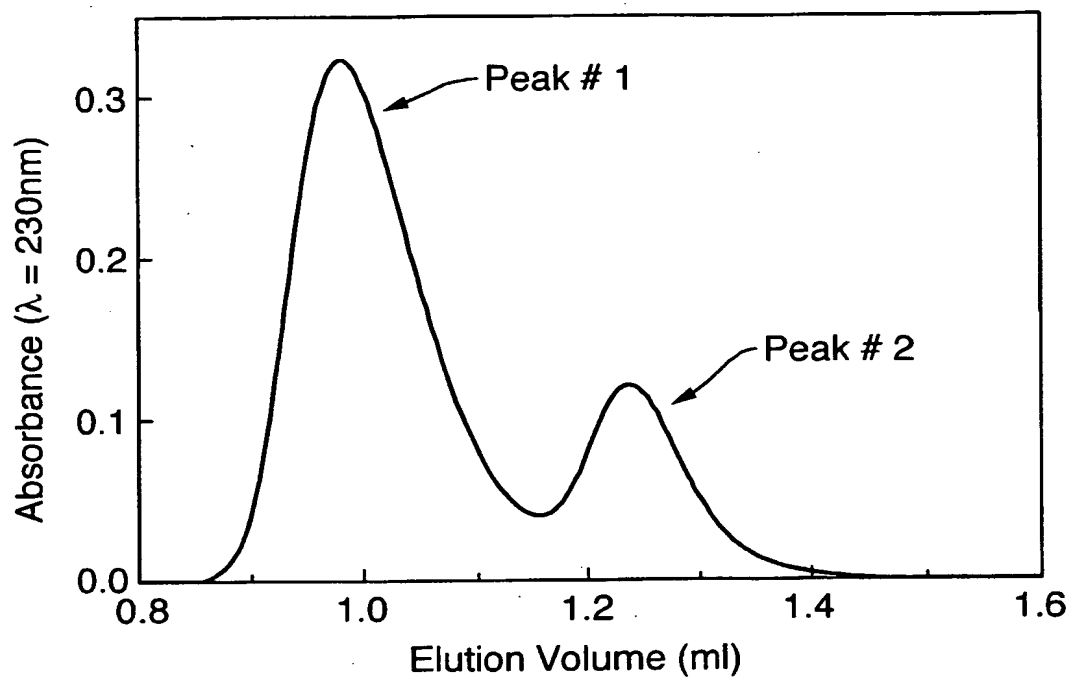
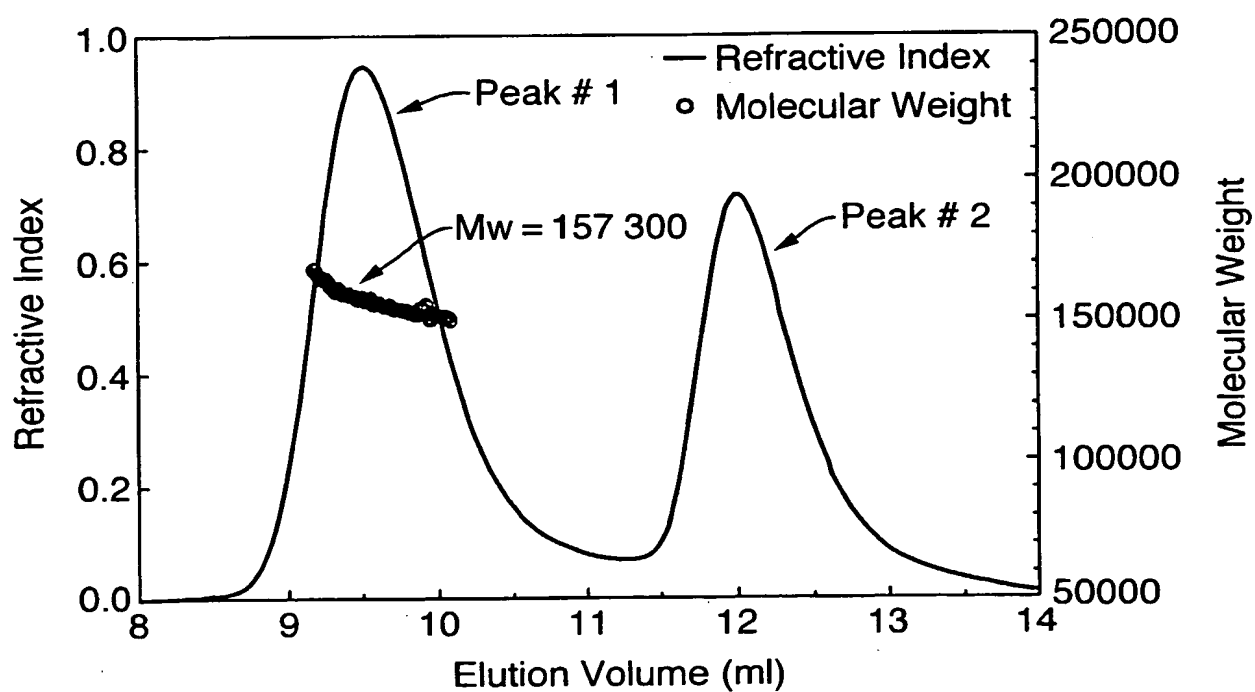


Fig.33.



SUBSTITUTE SHEET (RULE 26)

50/55

Fig.34.

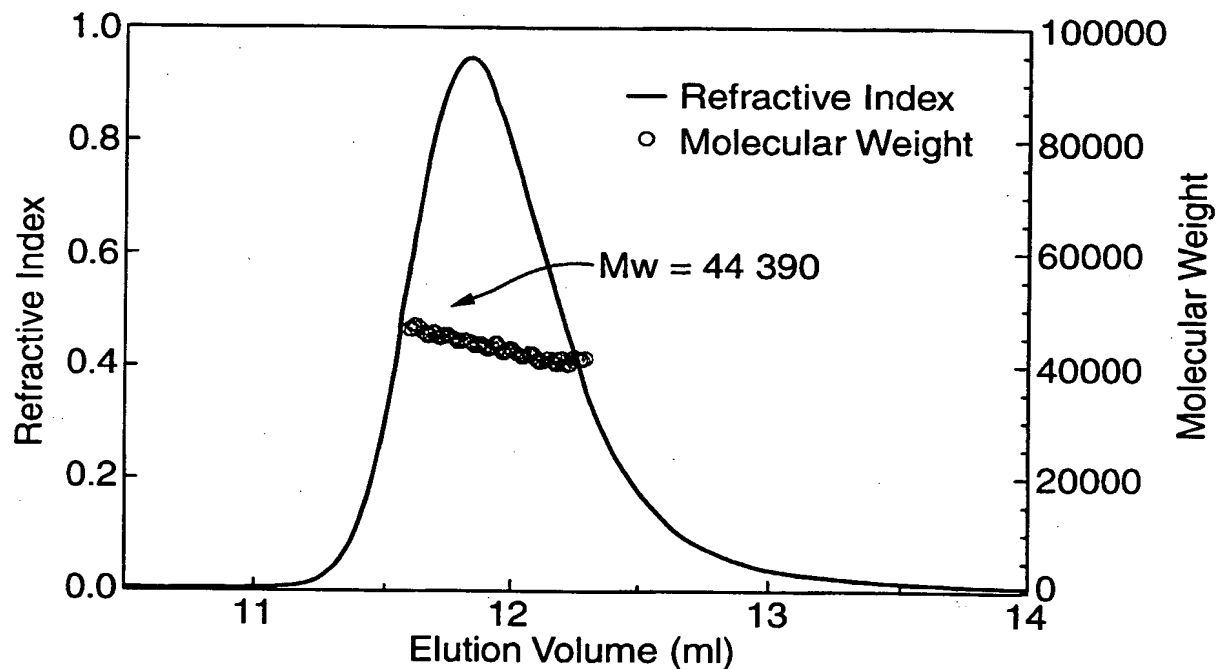
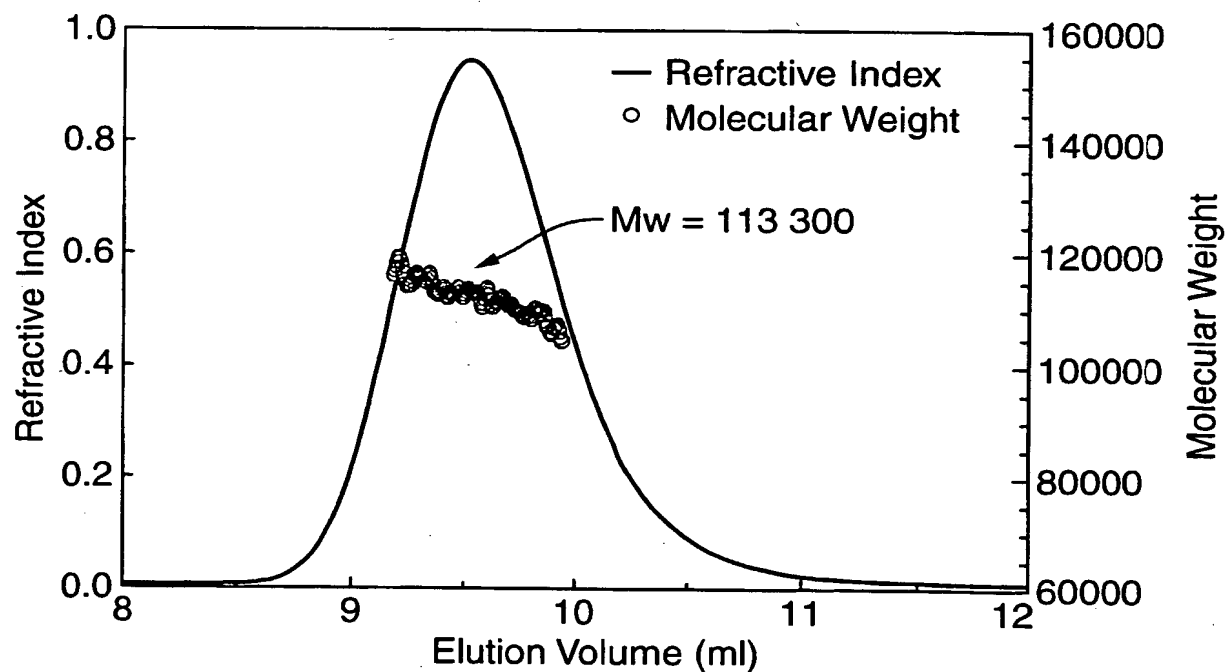


Fig.35.



SUBSTITUTE SHEET (RULE 26)

51/55

Fig.36.

50  
 GRPFVEMYSEIPEIIHMTGRELVIPCRVTSPNITVTLKKFPLDTLIPDG  
 100  
 KRIIWDNRKGFIIISNATYKEIGLLTCEATVNGHL YKTNYLTHRQNTIID  
 150  
 VVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNR  
 200  
 DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH  
 250  
 EKGPGDKTHTCPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD  
 300  
 VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN  
 350  
 GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL  
 400  
 TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKS  
 RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SUBSTITUTE SHEET (RULE 26)

52/55

Fig.37.

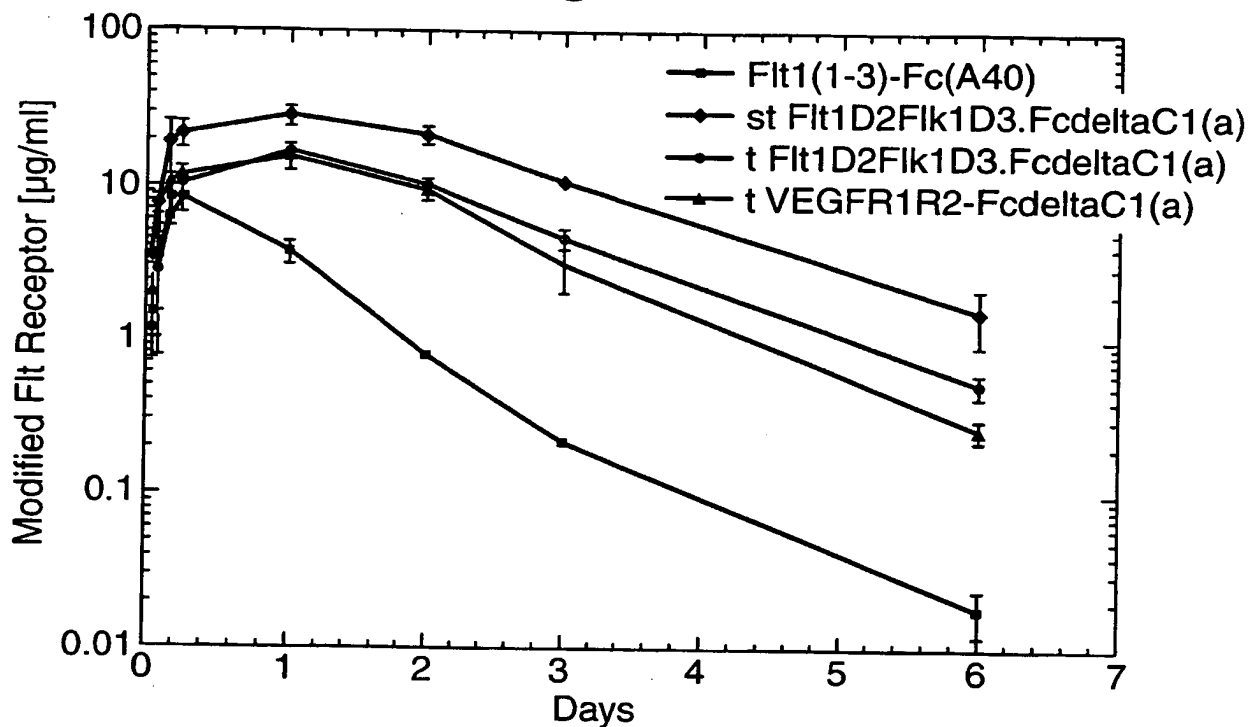
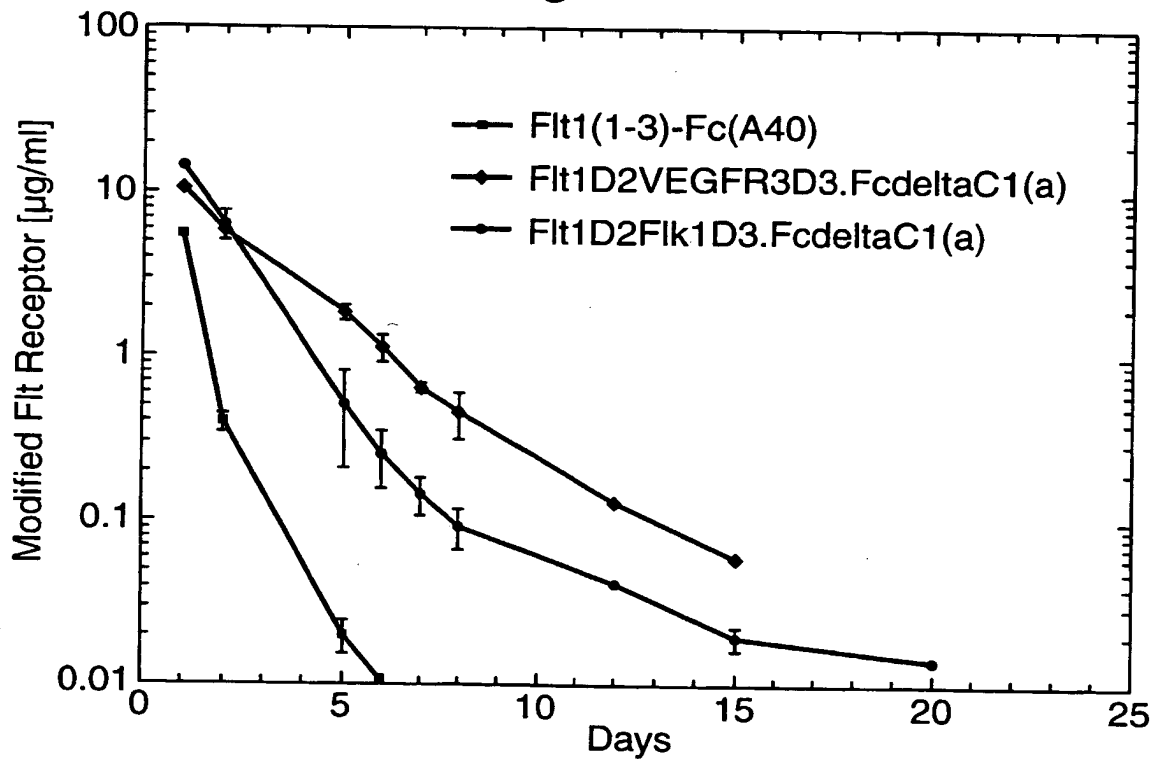


Fig.38.



SUBSTITUTE SHEET (RULE 26)



53/55

Fig.39.

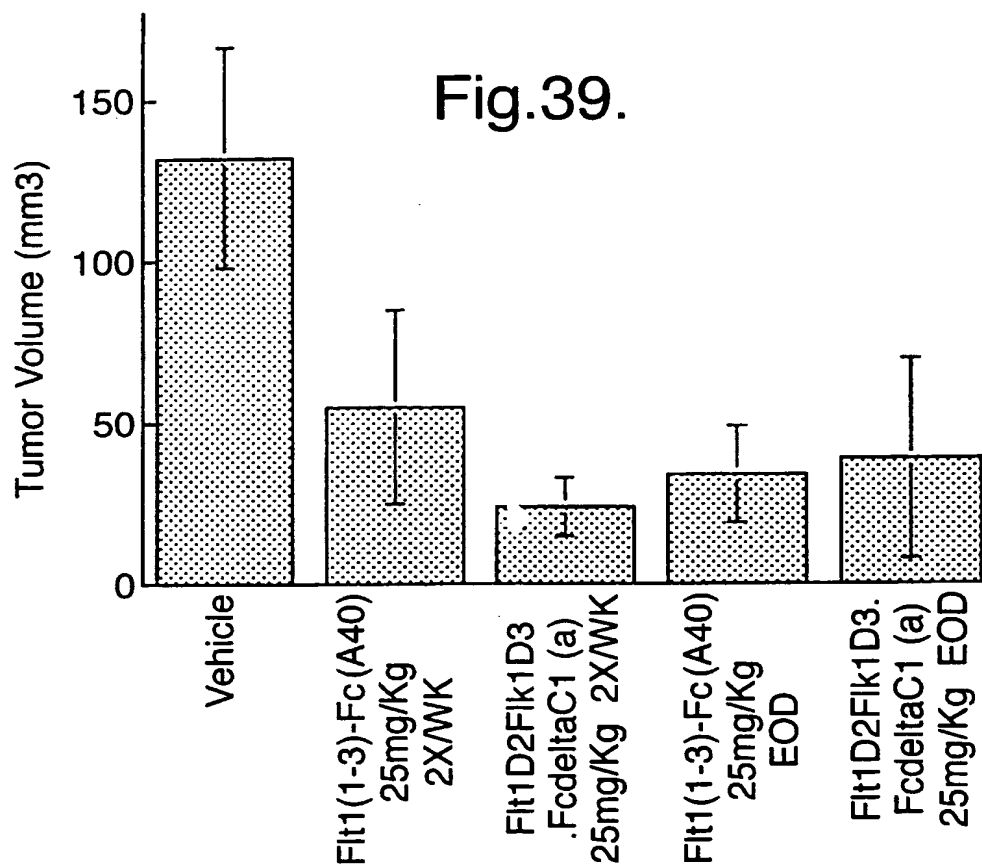
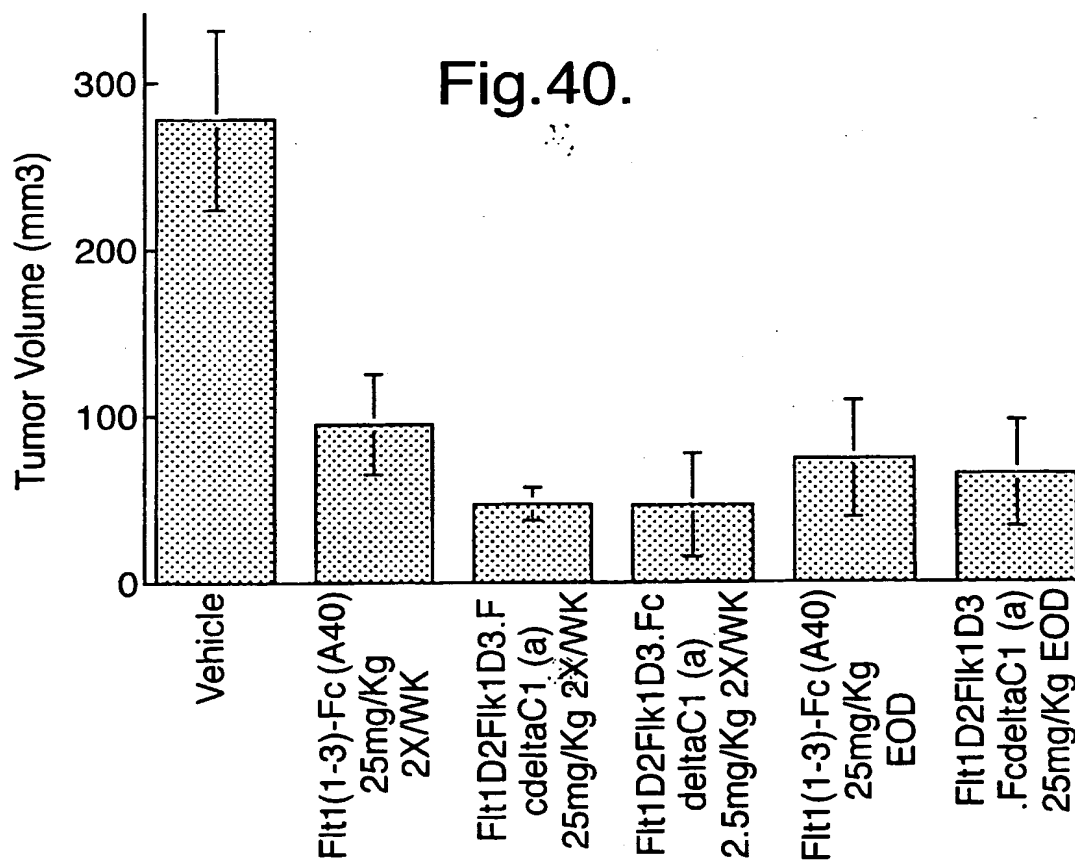
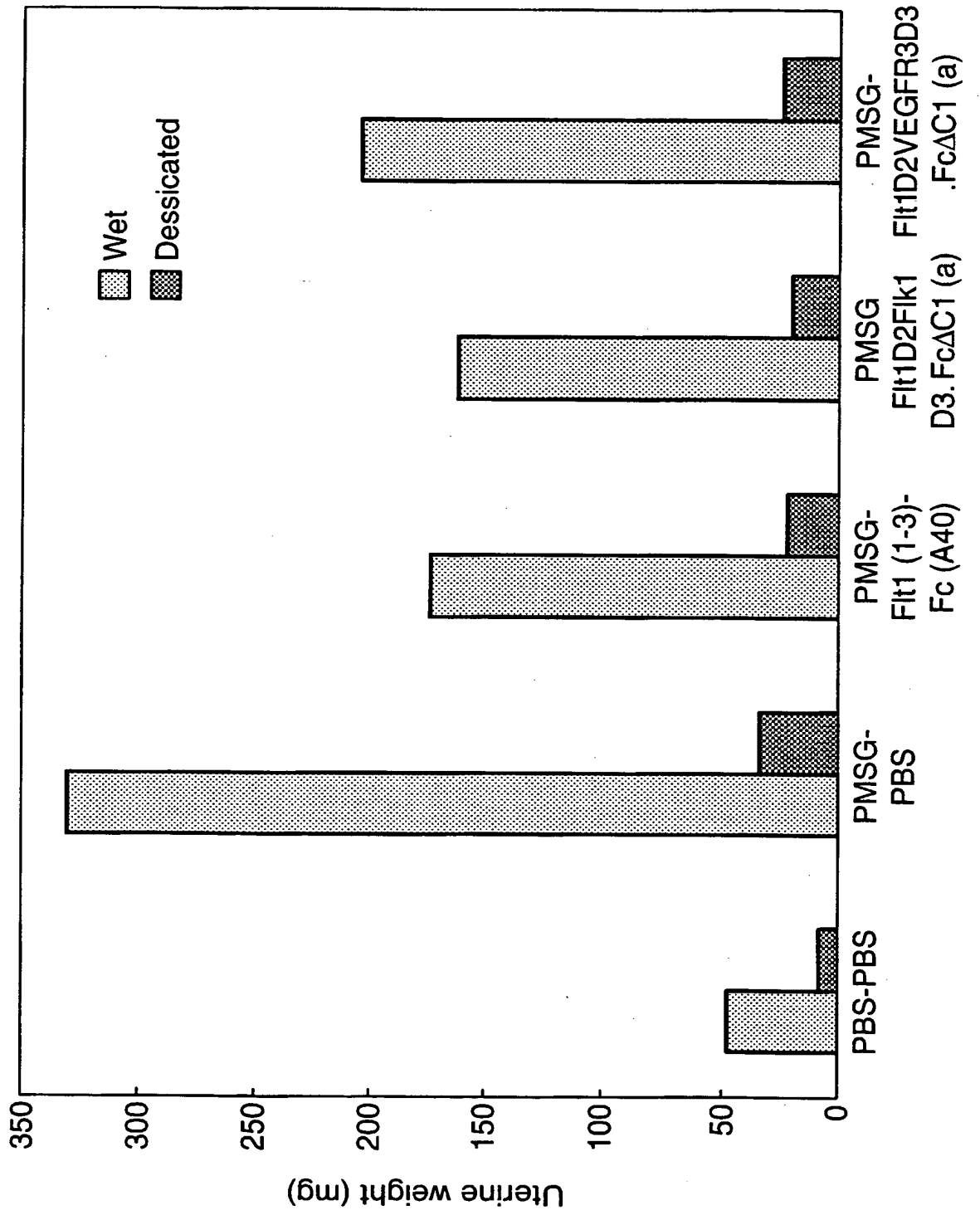


Fig.40.



SUBSTITUTE SHEET (RULE 26)

Fig.41.



55/55

Fig.42A.

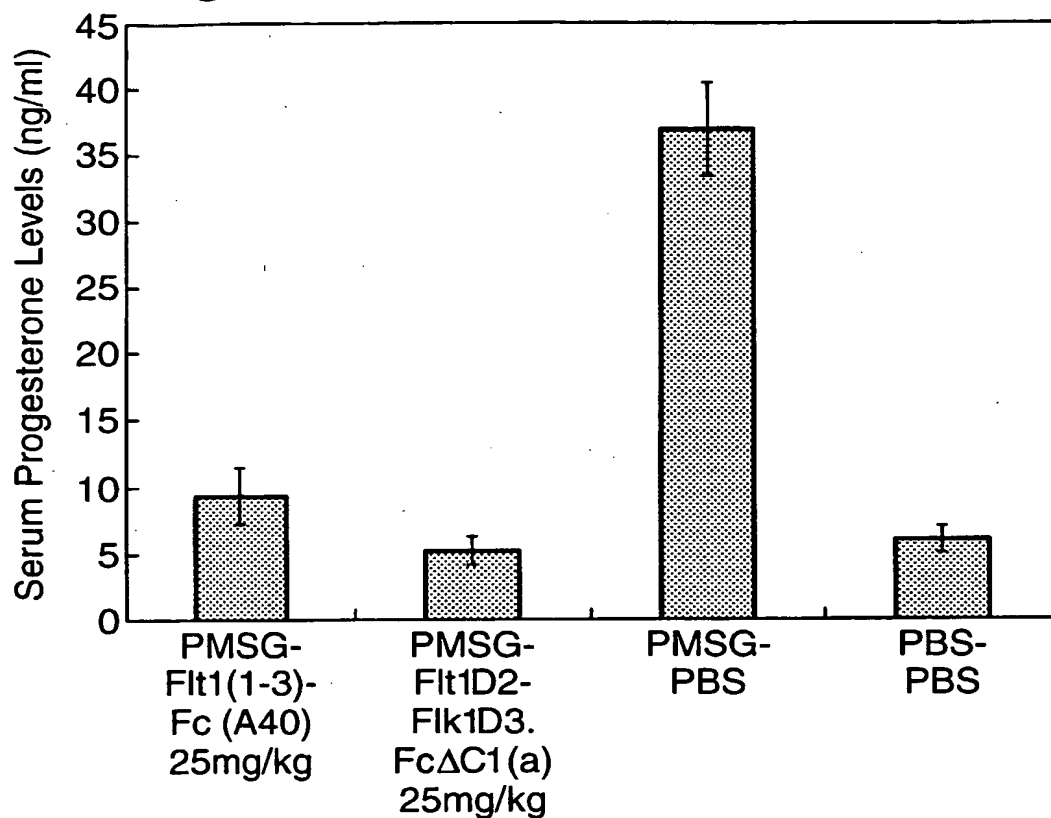
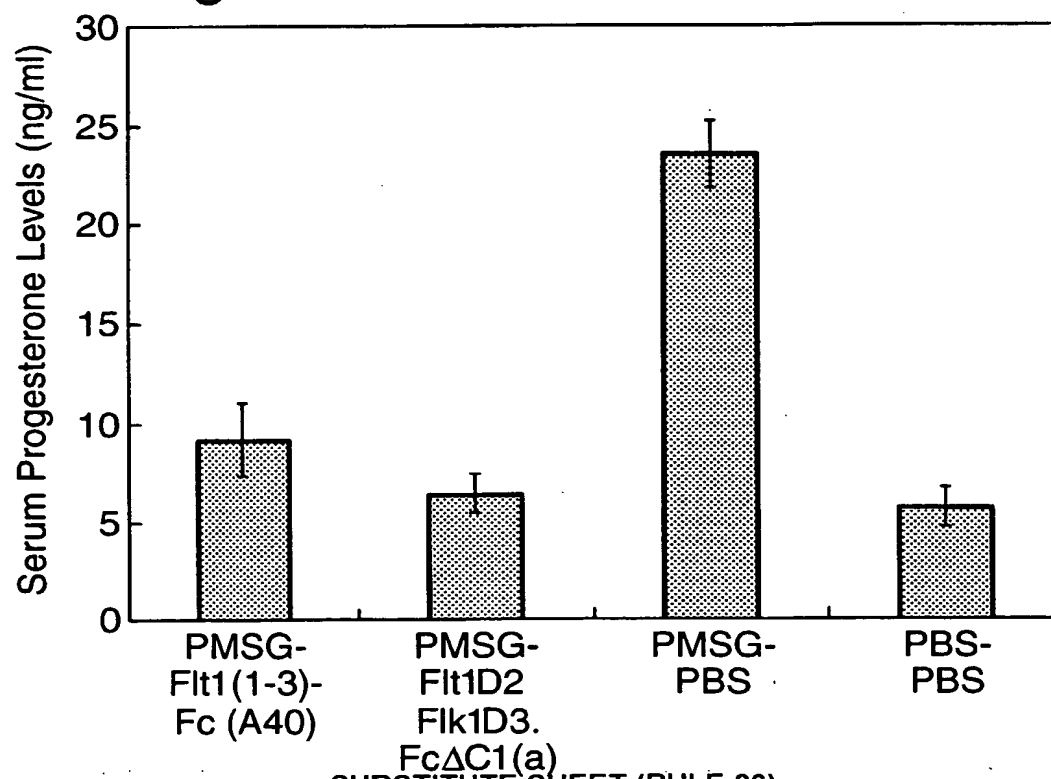


Fig.42B.



SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/14142

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/62 C12N5/10 C07K14/71 A61K38/17  
A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DAVIS-SMYTH T ET AL: "THE SECOND IMMUNOGLOBULIN-LIKE DOMAIN OF THE VEGF TYROSINE KINASE RECEPTOR FLT-1 DETERMINES LIGAND BINDING AND MAY INITIATE A SIGNAL TRANSDUCTION CASCADE" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 15, no. 18, 16 September 1996 (1996-09-16), pages 4919-4927, XP000611912 ISSN: 0261-4189 the whole document	1-21, 28-51
A	---	22-27
A	WO 97 44453 A (GENENTECH INC ;DAVIS SMYTH TERRI LYNN (US); CHEN HELEN HSIFEI (US)) 27 November 1997 (1997-11-27) cited in the application the whole document -----	1-51

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

30 August 2000

Date of mailing of the international search report

23. 11. 00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Galli, I

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 00/14142

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 28-31, 33-40, 50, 51 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
  
1-8, 41-48 and partly 9-40, 49-51

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-8, 41-48 and partly 9-40, 49-51

An isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising:

(a) a nucleotide sequence encoding a VEGF receptor component operatively linked to

(b) a nucleotide sequence encoding a multimerizing component,

wherein the VEGF receptor component is the only VEGF component of the fusion polypeptide and the nucleotide sequence (a) encodes essentially an Ig domain 2 of the extracellular domain of a first VEGF receptor and an Ig domain 3 of a second VEGF receptor.

Corresponding polypeptides, vectors, recombinant host cells, compositions, therapeutic applications. Where applicable, acetylated or pegylated polypeptides.

2. Claims: partly 9-21, 28-40, 49-51

An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of the nucleotide sequence depicted either in Fig 13A-D (seq. ID 3) or in Fig. 14A-C (Seq. ID 5).

3. Claims: partly 9-21, 28-40, 49-51

Idem as subject-matter 2, but limited to the sequence depicted in Fig.15A-C (Seq. ID 7).

4. Claims: partly 9-21, 28-40, 49-51

Idem as subject-matter 2, but limited to the sequence depicted in Fig.16A-D (Seq. ID 9)

5. Claims: partly 9-40, 49-51

A fusion polypeptide encoded by the nucleic acid sequence set forth in Fig. 10A-D (Seq. ID 1), which has been modified by acetylation or pegylation.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/14142

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9744453 A	27-11-1997	US 6100071 A	08-08-2000
		AU 717112 B	16-03-2000
		AU 3060497 A	09-12-1997
		EP 0907733 A	14-04-1999
		JP 2000502357 T	29-02-2000
		NZ 332779 A	29-06-1999
		US 5952199 A	14-09-1999
-----			

Form PCT/ISA/210 (patent family annex) (July 1992)

**THIS PAGE BLANK (USPTO)**



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**